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The Chromosomes and Transplantability of Tumors. II. Chromosome Duplication and the Loss of Strain Specificity in Solid Tumors[1]

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It has been known since 1926 (*1*) that there are some tumors that are not strain specific. These are different from strain-specific tumors and from nonmalignant tissues in that they do not necessarily require the same genotype in the transplanted tissue and in the host animal for successful transplantation. A wide range of transplantability can be obtained, even with strain-specific tumors, by altering the response of the host through treatment with such agents as cortisone (*2*) or lyophilized tissues (*3*, *4*). But tumors that are not strain specific are genetically capable of giving successful homotransplants, *i.e.*, transplants into animals belonging to the same species with different genotypes, without such treatment of the host.

Although the existence of tumors with this special property of successful homotransplantability has been known for 30 years, only recently has a combined approach to the study of the genetic basis of this phenomenon been utilized. This approach, which combines transplantation tests for strain specificity with an analysis of the chromosomes, has shown that a relationship can be found between the chromosome numbers of tumors and their transplantability (*5–7*), the absence of strain specificity being related to the presence of duplicated chromosomes in the fundamental cells of a tumor, *i.e.*, in those cells which determine the tumor's genetic behavior (*7*). Since duplication of chromosomes includes duplication of genetic loci, this therefore shows a relationship between the successful homotransplantation of a tumor and the presence of duplicate genetic loci (*7*).

The existence of this relationship originally was based on the comparison of chromosome numbers and strain specificity in different tumors, mainly ascites tumors of the mouse. This relationship was also shown by the result of Hauschka (*6*), who found one mouse ascites tumor which produced two sublines that differed from the original tumor both in their chromosome numbers and in their degree of strain specificity. After repeating this latter result in other tumors, the next step was to try to determine, in the same tumor, the immediate relationship between changes

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in strain specificity and in chromosome number and the presence of duplicate chromosome material by an examination of the chromosomes and transplantability at each successive transplant generation. This has been one of the main objects of the present investigation.

The previous studies on chromosomes and transplantability have dealt mainly with tumors in the ascites form, since tumors in this form are better both as experimental material and for chromosome analysis than tumors in the solid form. But from the point of view of the present experiments, this form of tumor has disadvantages, namely, that the existing ascites tumors (and this also applies to the solid tumors which have previously been studied) probably have been subject to considerable selection in their growth during many transplant generations, often because they have been grown in different substrains, different strains, or hybrid animals. The ascites tumors may also have been subject to selection when they were converted into the ascites form (*8*, *9*). Solid tumors studied in the same host substrain since the time of their production do not suffer from these disadvantages, and we have therefore used, for the present experiments, solid tumors that have arisen spontaneously or that we have induced experimentally in our own mice. With these tumors it is possible to begin with a population of cells that has not previously been subject to these types of selection, and the analysis may be begun from the time of tumor induction.

The present study deals with an analysis of the chromosomes and transplantability of 12 such solid tumors in the mouse, which were examined as far as possible at each successive transplant generation. Since the original chromosome constitution of a tumor is of importance for an understanding of its subsequent behavior and since there is very little previous data on the chromosomes of primary tumors, we have also included in the present investigation a chromosome analysis on 20 primary tumors of the mouse.

Materials and Methods

In the present studies we have used sarcomas and carcinomas which arose in our own mice. The sarcomas were produced by a subcutaneous injection of 2.5 mg. of benzpyrene in 0.25 cc. of tricapryllin, and the carcinomas arose spontaneously. Each tumor originated in a different mouse. All the tumors were transplanted intramuscularly with a trocar into the left leg.

The following strains of mice were used in our experiments: C3H, C57BL, C57BR/a, BALB/c, and Swiss. The C3H and C57BL were derived from inbred stocks obtained from Dr. W. E. Heston in 1951. Since then C3H mice have been mated brother × sister by us for at least eight generations and the C57BL mice for at least four generations. The Swiss mice were derived from an originally heterogeneous stock that we had mated brother × sister for at least eight generations, and the C57BR/a and BALB/c mice were obtained directly from the inbred colony of the Roscoe B. Jackson Memorial Laboratory.

The histologic examinations were made on sections from material fixed in Bouin's solution and stained with hematoxylin and eosin. The chromosome studies were made on Feulgen squashes (*10*). Small pieces of tumor were fixed, most frequently after pretreatment, in 45 percent acetic acid, stained by the ordinary Feulgen procedure, and then squashed in 45 percent acetic acid. The pretreatment method, which also had given good results with ascites tumors (*7*) and which greatly facilitated the chromosome analysis, consisted of immersing the pieces of tumor for about 20 to 30 minutes in a hypotonic salt solution before fixation. The chromosome studies were made mainly on temporary squash preparations, but some permanent slides were made from each examination by freezing the slide and cover slip on dry ice or liquid air, dehydrating, and mounting in euparal, as with the ascites tumors (*7*). Each time an analysis of the chromosomes was made, we examined several pieces from the same tumor, taking care to include pieces from those parts of the tumor that were used for transplantation.

The transplantability tests for tumors that originated in strain C3H mice were made at each transplant generation with a tumor taken from one C3H mouse. The transplantability tests for tumors that originated in Swiss, and in sublines of C3H tumors now being transplanted through Swiss, were made at each transplant generation with a tumor taken from one Swiss mouse. The chromosome analysis for each transplant generation was made on the tumor that was used for the transplantability tests.

Results

Classification of Chromosome Numbers

Tumors in the solid form are much less favorable material for chromosome analysis than tumors in the ascites form, and no solid tumor has yet been found which regularly gives the clear chromosome pictures that have been found in some ascites tumors. In the present chromosome counts, therefore, it has not been possible to obtain uniformly in all the tumors in all transplant generations the high degree of accuracy that can be obtained with ascites tumors, where nearly all chromosome counts can be made with a possible error that is at most ± 2, or even less.

The first difficulty of using solid tumors is that of making squash preparations with a solid, often hard, piece of tissue—a difficulty not encountered with a suspension of free cells, as in ascites tumors. Secondly, an ascites tumor can easily be sampled several times, if necessary, during growth in the same animal merely by removing some of the cell suspension from the peritoneal cavity. The best sample can then be taken for the chromosome analysis, whereas a solid tumor can be sampled only once from the same animal, unless special surgical procedures are employed. In the present experiments our desire to make an analysis as far as possible at each transplant generation created further difficulties, whereas most previous chromosome analyses on transplanted tumors have been made easier by using only the best samples taken during many transplant generations.

In order to obtain a standard classification which we could use for all our tumors throughout the experiments, we classified the present data on chromosome numbers in text-figures 1 to 4 and in table 1 into three groups: approximately diploid [2] (2n±), approximately triploid and tetraploid (3–4n±), and more than tetraploid (>4n). In all these analyses, there were only a very small proportion of cells whose chromosome numbers were about triploid, so that nearly all the cells in the 3 to 4n± group are about tetraploid. The 2n± group includes all the cells with approximately 35 to 50 chromosomes, and the >4n group includes all the cells with more than approximately 85 chromosomes.

In some transplant generations in some tumors the chromosomes could not be sufficiently separated, and it was even difficult to differentiate with certainty cells with 2n± from cells with 3 to 4n±. These transplant generations and, of course, those in which there were no mitoses have been omitted from text-figures 1 to 4. In addition to the data in text-figures 1 to 4, further information on the chromosome numbers of all the tumors studied is given in tables 2 and 3 and in the text. When the chromosome counts are given in numbers, *e.g.*, 45±, instead of degree of ploidy, *e.g.*, 2n±, they have a possible error of ±2 for 40± and 45±, and ±3 for 80±.

The Chromosomes of Primary Tumors

The chromosome numbers of 20 primary tumors that arose spontaneously or were induced experimentally in our C3H and Swiss mice are given in table 1. The data show that primary tumors from the same strain of origin and of the same general histologic type can differ in their chromosome constitution and can have a fundamental chromosome number of 2n±, 4n±, or both 2n± and 4n±.

TABLE 1.—*Chromosome numbers of primary tumors*

Tumor	Origin of tumor	Number of cells counted	Number of tumors	Percent of cells with chromosome Nos.:		
				2n±	3–4n±	>4n
Spindle-cell sarcoma.....	C3H*	20	1	60	35	5
Spindle-cell sarcoma.....	C3H*	12	1	50	33	17
Spindle-cell sarcoma.....	C3H*	15	1	0	93	7
Giant-cell sarcoma.....	C3H*	20	1	0	80	20
Mammary carcinoma.....	C3H†	20	7	100	0	0
Mammary carcinoma.....	C3H†	15	1	100	0	0
Mammary carcinoma.....	Swiss†	20	4	100	0	0
Mammary carcinoma.....	Swiss†	15	1	100	0	0
Mammary carcinoma.....	C3H†	20	1	75	25	0
Mammary carcinoma.....	Swiss†	15	1	47	53	0
Mammary carcinoma.....	C3H†	20	1	10	85	5

*Induced by a subcutaneous injection of benzpyrene.
†Spontaneous.

[2] 2n=40 in the diploid chromosome number of the mouse.

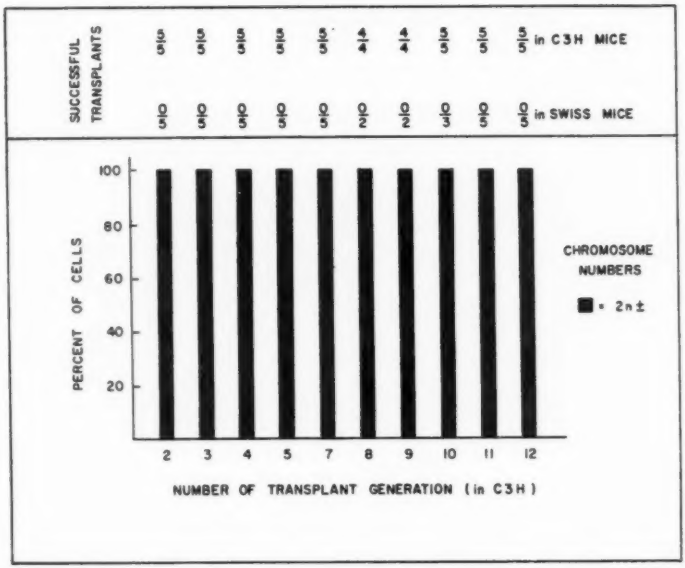
In one of the sarcomas and one of the carcinomas, the predominantly 4n± chromosome number of the primary tumor was changed during the first and subsequent transplant generations to a mixture of 2n± and 4n± cells. This is probably due to the fact that the 2n± cells were not dividing at the time when these primary tumors were examined, rather than to a change from a fundamental chromosome number of 4n± to a fundamental chromosome number of 2n±. It may therefore be necessary to measure the DNA content, which is not dependent upon the presence of mitoses, along with a chromosome analysis in order to establish the fundamental chromosome numbers of primary tumors.

A more detailed chromosome analysis than that made for table 1 has been carried out on three of the mammary carcinomas in which all the counted cells were in the 2n± group, and this has shown no greater range of chromosome numbers or chromosome and spindle abnormalities than that which can be found in nonmalignant somatic tissues. This emphasizes the point that the presence of great variations in chromosome numbers and chromosome and spindle abnormalities are not characteristic features of all malignant tissues (*11*).

Chromosomes and Strain Specificity in Successive Transplant Generations

In the present investigation we have studied the chromosomes and strain specificity as far as possible at each transplant generation in 12 solid tumors. These 12 tumors consist of 8 sarcomas—R1, R2, R4, R6, R12, R13, R14 and R19—and 2 carcinomas—W1 and W2—that originated in our C3H mice, and 2 sarcomas—R7 and R8—that originated in our Swiss mice. Since C3H and Swiss were the two strains of mice of which we had a sufficient number of animals, all the tumors were tested for transplantability in both these strains at each transplant generation. Transplantability tests were also made in C57BR/a, BALB/c, and C57BL mice whenever animals belonging to these strains were available. The *H-2* alleles (*12*) carried by the strains that we used are $H\text{-}2^K$ for C3H and C57BR/a, $H\text{-}2^D$ for BALB/c, $H\text{-}2^B$ for C57BL, and although we do not know exactly which *H-2* alleles are carried by our Swiss, it probably is not $H\text{-}2^K$. By using these different strains of mice, it was possible to determine the transplantability of a tumor that originated in C3H, in a foreign strain with the same *H-2* allele, and in foreign strains with different *H-2* alleles. For reasons which we have given in the Discussion, we have used capital letters instead of small letters for the superscripts, which indicate different *H-2* alleles.

Of the 12 tumors studied, there were 5 that gave the clearest chromosome figures during more than 10 transplant generations. The detailed data on the chromosome numbers in these 5 tumors in successive transplant generations and on their transplantability in C3H and Swiss are given in text-figures 1 to 4. Further data on the chromosomes and transplantability of these tumors in other strains of mice, and on the chromosomes and transplantability of the other 7 tumors are given in


CARCINOMA W1
SUCCESSFUL TRANSPLANTS
5/5 5/5 5/5 5/5 5/5 4/4 4/4 5/5 5/5 5/5 in C3H MICE
0/5 0/5 0/5 0/5 0/5 0/2 0/2 0/5 0/5 0/5 in SWISS MICE
PERCENT OF CELLS
100
80
60
40
20
2 3 4 5 7 8 9 10 11 12
NUMBER OF TRANSPLANT GENERATION (in C3H)
CHROMOSOME NUMBERS
2n±

TEXT-FIGURE 1.—The chromosome numbers and transplantability in C3H and Swiss mice of carcinoma W1. The percentages of cells are based on chromosome counts in 15 cells for transplant generations 7 and 10 and in 20 cells for all other generations. Further details on the chromosomes of this tumor and on its transplantability in other strains of mice are given in table 2 and the text.

tables 2 and 3 and the text. Photomicrographs of the chromosomes in some of the tumors are shown in figures 1 to 12 and histologic sections in figures 13 to 20.

The tumors that we studied during successive transplant generations can be divided into two main categories:

A) Tumors that have not lost their strain specificity (10 tumors).

B) Tumors that have lost their strain specificity (2 tumors).

A. Tumors That Have Not Lost Their Strain Specificity

The 10 tumors that belong to this category consist of 2 spontaneous mammary carcinomas, W1 and W2, 5 benzpyrene-induced spindle-cell sarcomas, R1, R4, R12, R13, and R6, and 1 benzpyrene-induced spindle- and giant-cell sarcoma, R19, that originated in our C3H mice; 1 benzpyrene-induced spindle-cell sarcoma, R7, and 1 benzpyrene-induced spindle- and giant-cell sarcoma, R8, that originated in our Swiss mice. Two of these tumors, R6 and R8, showed a temporary loss of strain specificity, but in neither case was this loss of specificity maintained on further transplantation.

Carcinoma W1.—The chromosome numbers and transplantability of the mammary carcinoma W1 are shown in text-figure 1 and in table 2. This

tumor has consistently maintained a chromosome number of 2n±, and it has never given successful transplants in a foreign strain during 18 transplant generations. Although the chromosomes of W1 were not clear enough for analysis in the primary tumor and the first transplant generation, chromosome counts on tumors in 4 C3H mice in each of the transplant generations 2, 3, and 4 have further confirmed the 2n± character of this carcinoma. A transplantability test in the first transplant generation gave 5/5 in C3H and 0/5 in Swiss.

Carcinoma W2.—This mammary carcinoma (table 2) had both 2n± and 4n± as its fundamental chromosome numbers. It has now maintained its strain specificity for five transplant generations and has given one negative transplant in the strain of origin in the first and in the second generation.

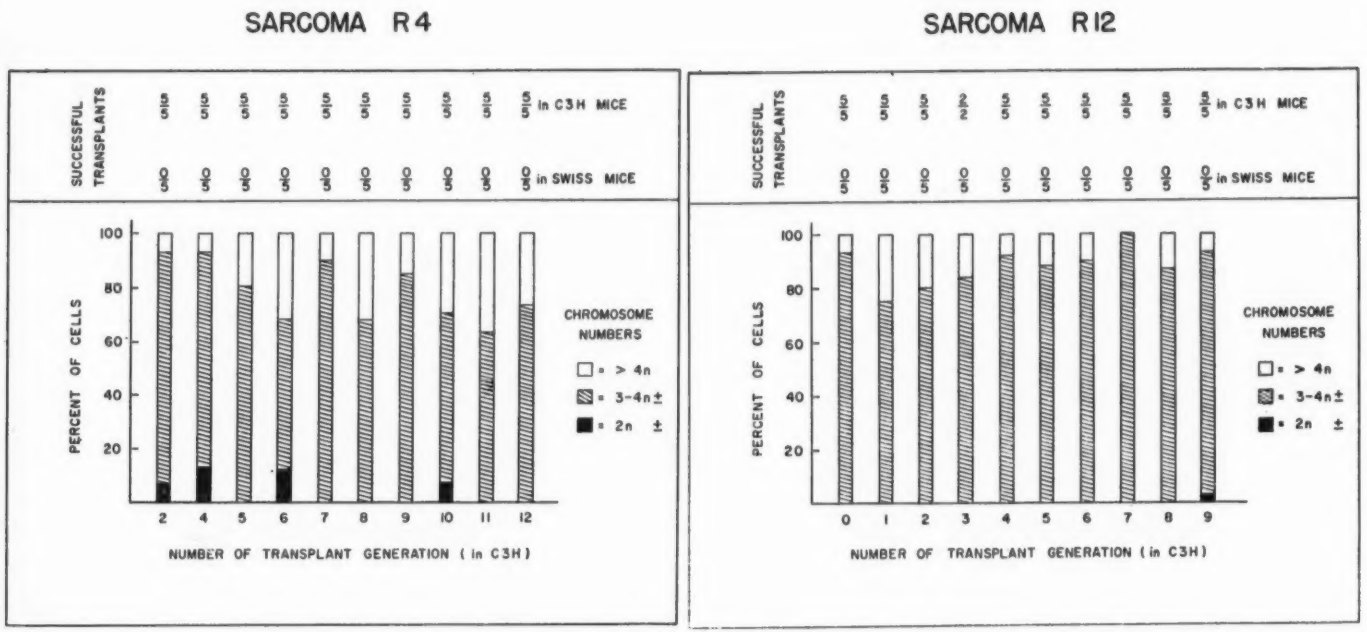
Sarcomas R1, R4, and R12.—These three spindle-cell sarcomas (text-fig. 2 and table 2) have all maintained their strain specificity with a fundamental chromosome number of 4n±. In R4, the chromosomes of the primary tumor and the first and third transplant generations were not sufficiently clear for analysis, but a transplantability test gave 5/5 in C3H and 0/5 in Swiss, both in the first and in the third generation.

Sarcomas R13 and R19 (table 2).—The spindle-cell sarcoma R13 had fundamental chromosome numbers of both 2n± and 4n±, with a predominance of 4n± cells. Sarcoma R19 began as a spindle- and giant-cell sarcoma, and the primary tumor gave one negative transplant in the strain of origin. The giant cells of R19 disappeared during transplantation, and this tumor is now a spindle-cell sarcoma with fundamental chromosome numbers of 2n± and 4n±, with a predominance of 2n± cells. R19 has now maintained its strain specificity for 5 transplant generations and R13 for 12 transplant generations.

TABLE 2.—*Transplantability of C3H tumors that have not lost their strain specificity*

Tumor	Chromosome numbers	Transplant generations	Total number of successful transplants in:				
			C3H	Swiss	C57BR/a*	BALB/c*	C57BL*
			H-2^K	*H-2$^?$*	*H-2^K*	*H-2^D*	*H-2^B*
Carcinoma W1..	40±	0–18	93/93	0/82	0/10	0/10	0/4
Carcinoma W2..	40± (and 80±)	0–5	28/30	0/30	0/5	0/5	0/5
Sarcoma R19...	40± (and 80±)	0–5	29/30	0/30	0/10	0/10	0/5
Sarcoma R13...	80± (and 40±)	0–12	65/65	0/65	0/5	0/5	0/5
Sarcoma R1....	80±	0–9	50/50	0/40	—	—	—
Sarcoma R4....	80±	0–12	62/62	0/60	0/5	0/5	0/5
Sarcoma R12...	80±	0–14	72/72	0/75	0/10	0/10	0/10
Sarcoma R6....	40± (and 80±)	0–16	83/83	5/85	0/10	0/10	0/4

*The transplantability tests in these strains of mice were made in the following transplant generations:
C57BR/a and BALB/c—generations 17 and 18 for W1; 5 for W2; 0 and 4 for R19; 12 for R13; 11 for R4; 9 and 14 for R12; and 15 and 16 for R6.
C57BL—generations 17 for W1; 4 for W2; 2 for R19; 11 for R13 and R4; 12 for R12; and 15 for R6.



TEXT-FIGURE 2.—The chromosome numbers and transplantability in C3H and Swiss mice of sarcomas R4 and R12. In *sarcoma R4*, the percentages of cells are based on chromosome counts in 30 cells for transplant generations 10, 11, and 12; in 25 cells for generations 6 and 8; in 20 cells for generations 7 and 9; and in 15 cells for generations 2, 4, and 5. In *sarcoma R12*, the percentages of cells are based on chromosome counts in 30 cells for transplant generations 8 and 9; in 25 cells for generations 3, 4, 5, and 7; in 20 cells for generations 1, 2, and 6; and in 15 cells for generation 0. Further details on the chromosomes of these tumors and on their transplantability in other strains of mice are given in table 2 and the text.

Sarcoma R7.—This spindle-cell sarcoma, which originated in one of our Swiss mice, had a fundamental chromosome number of 4n± and it was transplanted for nine generations. As we have pointed out in a previous section, our Swiss mice were not so highly inbred as the other strains of mice that we used; and even in this strain, R7 did not give successful transplants in all the mice into which it was transplanted. In C3H it gave a total of 0/39 transplants.

Sarcoma R8.—This spindle- and giant-cell sarcoma originated in one of our Swiss mice. Like R7, it had a fundamental chromosome number of 4n± and did not even give successful transplants in all the Swiss mice into which it was transplanted. R8 was transplanted for 18 transplant generations, during which time it was tested in a total of 95 strain C3H mice. Only in one transplant generation, the 10th, did it give successful transplants, in 2/5 C3H. On further transplantations of these two tumors growing in C3H, one gave 2/5 and one gave 1/5 successful transplants in C3H. The latter tumor was again transplanted, giving 3/5 in C3H; two of these three tumors were tested for further transplantability, and both gave 0/5 in C3H. Therefore, the loss of strain specificity by R8 was only a temporary one, and there were no further successful transplants in C3H mice.

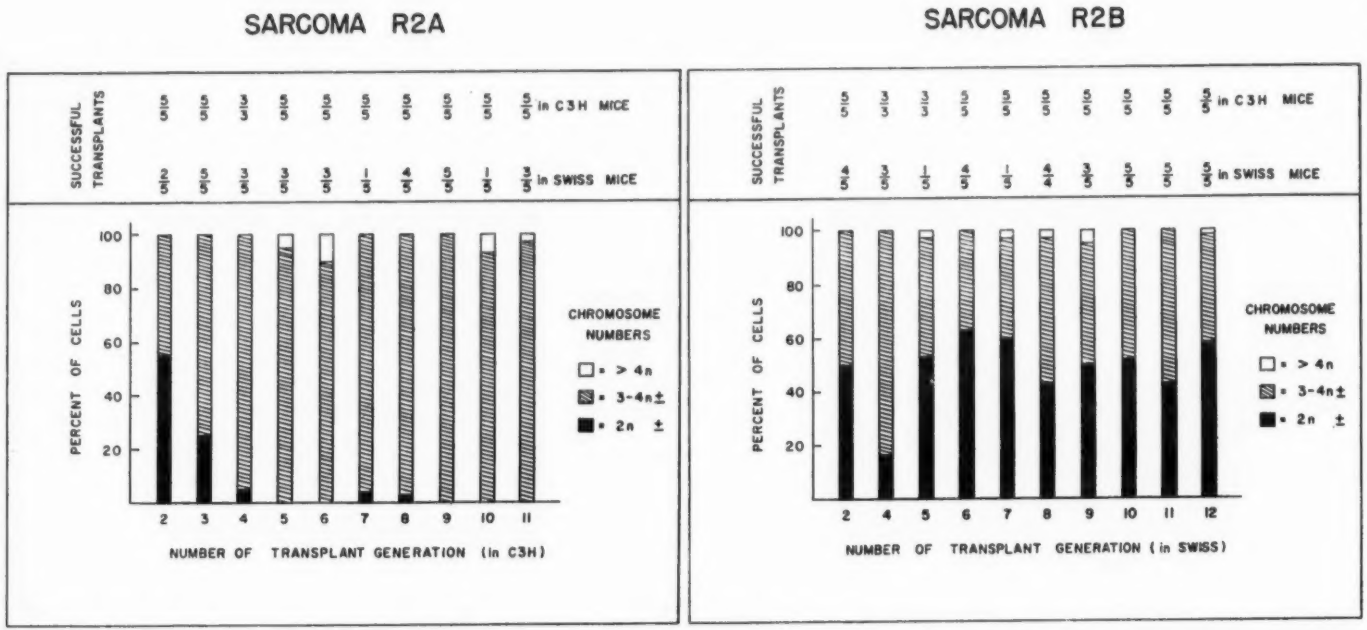
Sarcoma R6 (*table 2*).—This tumor originated in a C3H mouse and had both 2n± and 4n± as its fundamental chromosome numbers. It is now in its 16th transplant generation, with a predominance of 2n± cells. R6 has given a total of 5/85 successful transplants in Swiss mice, the 5 successful transplants consisting of 2/5 in the 4th and 3/5 in the 5th transplant generation. Both times, however, this loss of specificity was not maintained, since on both occasions further transplantation of one of the tumors growing in Swiss gave 0/5 in Swiss (and 5/5 in C3H). No further successful transplants in Swiss have been obtained since then, and when tested in other strains of mice, R6 gave no successful transplants in C57BR/a, BALB/c, and C57BL. The loss of strain specificity in R6 also was only temporary, and this tumor is now strain specific.

The two tumors, R8 with a fundamental chromosome number of 4n± and R6 with fundamental chromosome numbers of 2n± and 4n±, have therefore both shown only a temporary loss of strain specificity, and in neither tumor was this loss of specificity maintained on further transplantation.

Of the 10 tumors that did not lose their strain specificity, 1 has a fundamental chromosome number of 2n±; 5 have a fundamental number of 4n±; and 4 have fundamental numbers of both 2n± and 4n±—2 with a predominance of 2n± cells, and 1 with a predominance of 4n± cells.

B. Tumors That Have Lost Their Strain Specificity

The two tumors, R2 and R14, that have lost their strain specificity, are both benzpyrene-induced spindle-cell sarcomas that originated in our C3H mice, but R2 differs from R14 both in its transplantability and its chromosome constitution.



TEXT-FIGURE 3.—The chromosome numbers and transplantability in C3H and Swiss mice of sarcoma R2. In *sarcoma R2A*, the percentages of cells are based on chromosome counts in 30 cells for transplant generations 8, 10, and 11; in 25 cells for generations 7 and 9; and in 20 cells for all other generations. In *sarcoma R2B*, in which the 2n± category, especially in the later transplant generations, includes mainly cells with 45± chromosomes, the percentages of cells are based on chromosome counts in 50 cells for transplant generations 10 and 12; in 40 cells for generations 5, 7, 9, and 11; in 30 cells for generations 6 and 8; in 18 cells for generation 4; and in 12 cells for generation 2. Further details on the chromosomes of these tumors and on their transplantability in other strains of mice are given in table 3 and the text.

In both tumors, as soon as a successful transplant was obtained in Swiss mice, each tumor was divided into two sublines called R2A and R2B, and R14A and R14B. Both the A and the B sublines were always tested for transplantability at each transplant generation in C3H and in Swiss (and sometimes in other strains of mice); R2A and R14A were maintained by transplanting in C3H mice, whereas R2B and R14B were maintained by transplanting in Swiss mice. It has thus been possible to study the effects of selection on R2 and R14 during an extended period of growth in the originally foreign Swiss and to compare these selected tumors with those that have always grown in the strain of origin. In both R2 and R14, the loss of strain specificity was observed the second time the tumor was transplanted. In both cases, therefore, the first transplant generation taken from Swiss mice was numbered transplant generation #2B to correspond with transplant generation #2A, since this was the second transplant generation of the A subline of the tumor.

Sarcoma R2.—The chromosome numbers and transplantability of R2 are shown in text-figure 3 and table 3. Comparison of R2A with R2B shows that continued transplantation in Swiss mice, in contrast to continued transplantation in the strain of origin, has produced a striking difference between the two sublines of this tumor. Both R2A and R2B are now in transplant generation #16, but R2A now has a fundamental chromosome number of 80± and R2B now has a fundamental chromosome number of 45±.

In the primary tumor and first transplant generation of R2, the chromosomes were not sufficiently clear for analysis, but a transplantability test in the first generation gave 5/5 in C3H and 2/5 in Swiss. The primary tumor unfortunately was tested only in C3H mice, so that R2 may not have been strain specific from the very beginning. In R2B, the chromosomes in the third transplant generation were not sufficiently clear for analysis, but a transplantability test gave 5/5 in C3H and 4/5 in Swiss.

Although R2A has always been transplanted from mice belonging to the strain of origin, this tumor has always given some successful transplants in Swiss mice since it was first tested in the first transplant generation, and its transplantability in Swiss is now only a little below that of

TABLE 3.—*Transplantability of C3H tumors that have lost their strain specificity*

Tumor	Chromosome numbers	Transplant generations	Total number of successful transplants in:				
			C3H	Swiss	C57BR/a*	BALB/c*	C57BL*
			H-2^k	*H-2^s*	*H-2^k*	*H-2^d*	*H-2^b*
Sarcoma R2A	80±	0–16	84/84	68/105	13/15	12/15	5/5
Sarcoma R2B	45±	2–16	71/71	89/104	24/25	22/25	8/8
Sarcoma R14A	80±	0–14	75/75	10/100	10/25	0/25	0/8
Sarcoma R14B	80±	2–14	65/65	86/90	15/15	15/15	4/4

*The transplantability tests in these strains of mice were made in the following transplant generations:
C57BR/a and BALB/c—generations 12 and 16 for R2A; 10, 14, 15, and 16 for R2B; 9, 12, 13, and 14, for R14A; and 9, 13, and 14 for R14B.
C57BL—generations 14 for R2A; 12 and 14 for R2B; 13 and 14 for R14A; and 12 for R14B

R2B. Thus, in addition to the data in text-figure 3, tests on more than 5 Swiss mice per generation gave 13/20 in the 14th, 8/10 in the 15th, and 7/10 in the 16th transplant generation for R2A, in contrast to 10/10 in the 14th, 15th, and 16th transplant generations for R2B. Since the 10th transplant generation, R2B has always given 100 percent successful transplants in Swiss mice. The total transplantability (table 3) is now 65 percent for R2A and 86 percent for R2B in Swiss mice. When tested in other strains of mice (table 3), both R2A and R2B gave a high percentage of successful transplants in C57BR/a, BALB/c, and C57BL.

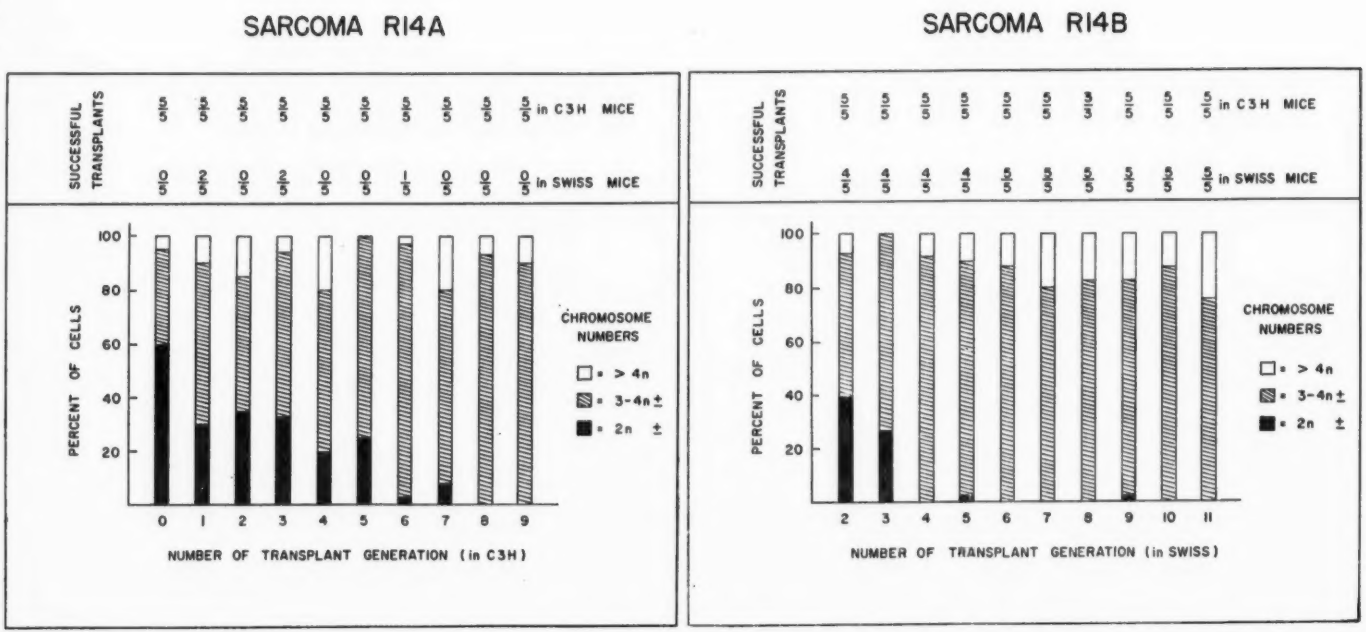
R2A originally had fundamental chromosome numbers of both 2n± and 4n±, and this then changed to a fundamental number of only 4n± (80±). Chromosome counts in the 4th transplant generation on 2 tumors growing in Swiss and in the 9th, 10th, and 11th generations on one tumor growing in Swiss have shown that the fundamental number of 80± is also present in R2A tumors growing in Swiss mice.

R2B now has a fundamental chromosome number of 45±. The 2n± category of R2B in text-figure 3, especially in the later transplant generations, includes mainly cells with 45± chromosomes, and the 3 to 4n± category includes cells with 90± chromosomes. The 45± cells have been selected from a population of different chromosome numbers which originally included many 80± cells (*e.g.*, transplant generation #4). Chromosome counts in the 10th transplant generation on 2 tumors growing in C3H and on 1 tumor growing in BALB/c, have shown that the fundamental number of 45± is also present in R2B tumors growing in these strains of mice. There is, therefore, a striking difference between R2A and R2B in their chromosome constitution but only a slight difference in their transplantability.

Sarcoma R14.—The chromosome numbers and transplantability of R14 are shown in text-figure 4 and table 3. In contrast to sarcoma R2, both R14A and R14B now have a fundamental chromosome number of 4n± (80±), and R14A differs strikingly from R14B in its transplantability.

Chromosome counts on one tumor growing in C3H in the eighth transplant generation and on two tumors growing in C57BR/a and BALB/c in the ninth transplant generation have shown that the fundamental number of 80± is also present in R14B tumors growing in these strains of mice.

In R14B, the continued transplantability of the tumor in the originally foreign Swiss mice has given 80 percent successful transplants since the first transplant generation and 100 percent successful transplants since the sixth transplant generation. On the other hand, R14A, which has been continuously transplanted in the strain of origin, has only occasionally given successful transplants in Swiss mice. Both R14A and R14B are now in transplant generation #14, with a total of 10 percent successful transplants in Swiss for R14A and 96 percent successful transplants in Swiss for R14B (table 3). In transplantability tests involving more than 5 Swiss mice per generation, R14B gave 20/20 in the 12th and 10/10 in the 13th and 14th transplant generations.



TEXT-FIGURE 4.—The chromosome numbers and transplantability in C3H and Swiss mice of sarcoma R14. In *sarcoma R14A*, the percentages of cells are based on chromosome counts in 30 cells for transplant generations 4, 6, and 9; in 25 cells for generation 7; in 20 cells for generations 0, 1, 2, and 5; and in 15 cells for generations 3 and 8. In *sarcoma R14B*, the percentages of cells are based on chromosome counts in 35 cells for transplant generation 9; in 30 cells for generations 2, 5, 7, and 8; in 25 cells for generations 4, 6, 10, and 11; and in 15 cells for generation 3. Further details on the chromosomes of these tumors and on their transplantability in other strains of mice are given in table 3 and the text.

In addition to the data in text-figure 4, R14A has also given some further successful transplants in Swiss mice in the 11th (4/5) and 12th (1/20) transplant generations, in contrast to 0/5 in the 10th and 0/10 in the 13th and 14th generations. Therefore, the successful transplantation of R14A in Swiss has always been less than 100 percent, and successful transplants were never obtained consecutively for more than two transplant generations. R14A gave no successful transplants in BALB/c and C57BL mice (table 3). But in C57BR/a, a strain of mice with the same *H-2* allele as the strain in which this tumor originated, R14A gave 0/5 in the 9th, 5/5 in the 12th, 4/5 in the 13th, and 1/10 in the 14th transplant generation. R14B (table 3), however, gave a high percentage of successful transplants in C57BR/a, BALB/c, and C57BL mice.

There are, therefore, striking differences between R2 and R14, the two sarcomas that have lost their strain specificity. R2A now has a fundamental chromosome number of 80±, and R2B, a fundamental number of 45±. Both R2A and R2B have given a high percentage of successful homotransplants in a strain of mice with the same *H-2* allele as the strain in which the tumor originated and also in strains of mice with other *H-2* alleles. Both R14A and R14B, on the other hand, now have a fundamental chromosome number of 80±. In R14A, a high percentage of successful homotransplants has never been obtained for more than two successive transplant generations in any of the strains of mice that were tested. But in R14B, a high percentage of successful homotransplants has been obtained, both in a strain of mice with the same *H-2* allele as the strain in which this tumor originated and in strains of mice with other *H-2* alleles.

Time Intervals between Transplant Generations

The time intervals between transplant generations were chosen on the basis of the time taken by the tumors to kill their hosts and also on the time available for carrying out the experiments. Table 4 shows, for up to 12 transplant generations, the intervals (in days) for the 10 tumors that had been transplanted for 9 or more generations. The intervals for the 2 tumors transplanted for 5 generations were 62, 43, 54, 44, and 52 days, respectively (mean=51), for carcinoma W2, and 38, 29, 31, 30, and 26 days, respectively (mean=31), for sarcoma R19.

Since the time intervals in each of the tumors varied between successive transplant generations, a comparison can be made between time intervals and transplantability. This comparison shows that the differences in the age of the tumors at transplantation did not produce differences in transplantability. There also appears to be no constant relationship, in these data, between the age of the tumors at transplantation and changes in fundamental chromosome numbers.

Survival Times of Host Animals

The mean survival times of the host animals, for the tumors listed in table 4, are shown in table 5. Carcinoma W1, with a fundamental chromosome number of 40±, takes longer to kill than any of the sarcomas.

TABLE 4.—*The time intervals between transplant generations**

Tumor	Strain of origin	Strain used for transplantation	Time interval for transplant generation												Mean time interval
			1	2	3	4	5	6	7	8	9	10	11	12	
Sarcoma R6	C3H	C3H	36	40	33	35	36	28	34	35	28	34	39	32	34
Sarcoma R12	C3H	C3H	27	42	40	18	48	36	36	39	29	21	34	30	33
Sarcoma R13	C3H	C3H	48	33	31	39	40	44	39	27	35	31	19	22	34
Sarcoma R1	C3H	C3H	31	32	21	28	29	21	36	40	60	—	—	—	33
Sarcoma R7	Swiss	Swiss	25	27	43	33	33	49	22	44	44	—	—	—	36
Sarcoma R8	Swiss	Swiss	24	24	19	34	21	22	23	20	19	25	31	21	24
Sarcoma R4	C3H	C3H	27	70	32	50	44	51	57	50	81	34	22	29	46
Carcinoma W1	C3H	C3H	26	24	27	29	50	45	31	39	39	46	57	67	40
Sarcoma R2A	C3H	C3H	40	32	38	47	41	44	33	48	42	28	30	30	38
Sarcoma R2B	C3H	Swiss	—	32	32	56	51	70	62	44	46	41	29	31	45
Sarcoma R14A	C3H	C3H	38	30	37	38	37	38	35	29	39	36	30	24	34
Sarcoma R14B	C3H	Swiss	—	67	27	27	34	29	29	29	31	37	27	27	33

*The time intervals are given as number of days between each transplant generation.

Among the sarcomas, there is a difference of 14.1 days between the 80± sarcomas R12 and R4 and only a difference of 5.3 days between the 80± sarcoma R12 and the predominantly 40± sarcoma R6. The mean survival time was, therefore, not related to the fundamental chromosome numbers of these tumors.

TABLE 5.—*The mean survival times*

Tumor	Host	Survival time (*days*)*	
		Mean	*S. E.* ±
Sarcoma R6	C3H	35. 0	0. 3
Sarcoma R12	C3H	40. 3	1. 3
Sarcoma R13	C3H	40. 6	2. 0
Sarcoma R1	C3H	42. 8	1. 5
Sarcoma R7	Swiss	47. 3	2. 4
Sarcoma R8	Swiss	51. 3	3. 1
Sarcoma R4	C3H	54. 4	2. 2
Carcinoma W1	C3H	70. 0	3. 4
Sarcoma R2A	C3H	42. 7	1. 5
	Swiss	58. 1	2. 6
Sarcoma R2B	C3H	51. 3	1. 8
	Swiss	51. 9	2. 4
Sarcoma R14A	C3H	49. 0	1. 5
Sarcoma R14B	C3H	36. 9	1. 4
	Swiss	50. 0	3. 4

*The mean survival times of the host animals are based, in sarcoma R7, on the first 15 animals killed by the tumor, and in all the other tumors on the first 30 animals killed by the tumor.

In both sarcomas that have lost their strain specificity, the loss was not correlated with a more rapid killing time of the tumor in the foreign host. R14A in C3H and R14B in Swiss have an almost identical mean survival time as that of the hosts. In R2 the most rapidly killing tumor was R2A in C3H, whereas in R14 the most rapidly killing tumor was R14B in C3H. In both R2 and R14 there were differences in the mean survival times of the A and B sublines. R2A killed more rapidly in C3H than in Swiss, whereas R2B had an almost identical mean survival time in C3H and in Swiss. R14B killed more rapidly than R14A in C3H, whereas R2B killed more slowly than R2A in C3H and more rapidly than R2A in Swiss.

Discussion

Fundamental Tumor Cells

In the following discussion it is, of course, understood that cells may have different genetic constitutions without any observable morphologic differences in the mitotic chromosomes, and that, when evaluating data on chromosome numbers, the important point is not so much the number itself but how far differences in chromosome number represent differences in chromosome material and, thus, differences in genetic material. Some indications of how far differences in chromosome number represent observable differences in the amount and arrangement of chromosome material can be obtained by an analysis of the morphologic appearance of

the chromosomes. Although some obvious morphologic differences have been found between different tumors, it will be difficult to determine small differences in the chromosomes of the mouse, since the normal mitotic chromosomes of this species have almost no good cytologic markers (*13*). For this purpose, the introduction of other laboratory animals such as the striped (Chinese) hamster (*14*) or some of the marsupials (*15*), which have a low chromosome number and some very clear cytologic markers on the chromosomes, should prove especially useful.

In discussing the concept of fundamental tumor cells it should also be emphasized first that, although some tumors show a great variation in chromosome number and a variety of chromosome and spindle abnormalities, some of the spontaneous mammary carcinomas examined in the present study are examples of tumors with no such chromosome variation and no such abnormalities. Although growth in tissue culture cannot be strictly compared to growth in the body, a chromosome count on a human melanoma (*16*) has shown that a tumor growing in tissue culture can have chromosome numbers that are very similar to those of some nonmalignant tissues growing in tissue culture. The existence of a great variation in chromosome number, and of a variety of abnormalities, is therefore not a characteristic feature of all malignant tissues. There are, in fact, nonmalignant tissues such as deciduomas (*17*), in which probably all the cells eventually become polyploid, in contrast to malignant tumors such as our carcinoma W1, in which there was not a single polyploid cell in a chromosome count on 250 cells during 12 transplant generations. Although a high frequency of cells with less than the diploid number is not found in any nonmalignant tissues (*11*, *18*), this is also a peculiarity found only in some tumors.

The concept of fundamental tumor cells arose out of studies on tumors with a range of chromosome numbers. These tumors, because of their variation in chromosome number, contain a variety of cells with different genetic constitutions, so that it is necessary to ascertain the role of these different cells in determining the tumors' genetic behavior. As a first step in the analysis of this problem, studies were made which showed that each tumor with a range of chromosome numbers has a predominant number which could, in the cases examined, maintain its predominance during many transplant generations under different environmental conditions. In addition, transplantability tests have shown a relationship between the predominant chromosome number of a tumor and its transplantability into hosts of different genetic constitution. This demonstrated that it is the cells with the predominant chromosome number that determine a tumor's genetic behavior. The predominant chromosome number in a tumor was therefore called the fundamental number, and the cells with the fundamental number were called the fundamental cells (*7*).

Evidence in support of the concept of fundamental cells has also been obtained by studies on the effects of chemicals, in which it has been shown, in 2 rat tumors, that the fundamental cells are more resistant than the

other cells of the tumor to podophyllin and to a $CaCl_2$ solution [3] (*19*, *20*).

It may also be possible to obtain evidence supporting the concept of fundamental tumor cells establishing tumors from single isolated cells (*6*, *21*, *22*). But since it has already been shown that not all tumors can be established with the same ease, either from a single cell or from a small number of cells (*6*) (some tumors could not be established at all by this method), these isolation experiments will pick out cells that can establish a tumor from a single cell, in contrast to cells that are either unable or less likely to establish a tumor in this way. This could explain the preferential recovery of certain chromosome numbers which have been found in mouse tumors obtained from single-cell isolations (*6*, *21*). Single isolated cells are also different from the same cells produced singly in a growing tumor, since in a growing tumor there can be an interaction between neighboring cells. Therefore, it is not yet clear whether the ability to establish a tumor from a single isolated cell is necessarily a property of the fundamental cells of a tumor, but further isolation experiments should be able to clarify this point.

Although some of the tumors that we have studied have maintained the same fundamental number during many transplant generations, the concept of fundamental tumor cells does not mean that the same fundamental number is always maintained in a tumor. This is clear from the fact that, in some tumors, cells that deviate from the existing fundamental cells have been able to establish themselves as a separate cell lineage. This can then bring about, as in our sarcoma R2, a change in the fundamental chromosome number of the tumor, or, as in our carcinoma W2, a tumor with two different fundamental numbers at the same time. There may also be a balance between two (or possibly more) similar chromosome numbers in the same tumor (*23*).

The possibilities of such genetic variation in a tumor therefore raises the question of the potentialities of cells that deviate from the existing fundamental cells. It is clear that all cells that deviate from the existing fundamental cells cannot automatically be classified as useless by-products (*11*), since what may at one moment of time be a derivative of the existing fundamental cells may, if it has the advantages for survival, appear at another time as the fundamental chromosome number. It is possible, however, by an analysis of the fundamental numbers found in different tumors, to determine the range of chromosome numbers that can establish themselves as fundamental cells.

In the mouse, the fundamental numbers that have been found to maintain themselves in the present and other investigations (*5–7*, *21*, *23–27*) range from 40 to 92. The highest numbers recorded are 90 to 92 chromosome tumors, produced by experimental chromosome doubling of a tumor with 45 or 46 chromosomes (*26*). Whether 92 is the upper limit in the mouse, and whether the establishment of higher chromosome numbers can be effected, in spite of possible difficulties at mitosis, could be deter-

[3] If this resistance to chemical (and/or physical) agents is found to be a characteristic of the fundamental cells of other tumors, this would be important for studies in tumor therapy.

mined by attempting experimental chromosome doubling of tumors with more than 46 chromosomes.

One tumor has been reported in the mouse, an ovarian teratoma (*27*), with a fundamental number of 38—two less than the normal diploid number of the mouse—in the early transplant generations, but this then changed to a fundamental number of 42, *i.e.*, two more than the diploid number. This tumor is peculiar in that the chromosomes show strong somatic pairing, a fact that would agree with one of the possible origins of teratomas, which can be determined by the diagnosis of the sex chromosomes in somatic tissues (*28*), and this pairing, which has not been found in any of the other tumors studied (since none of these are teratomas), may produce special types of chromosome combinations. Whether the cells with 38 chromosomes have chromosome rearrangements and an appreciably smaller amount of chromosome material than the diploid complement of the mouse is presumably being studied. But the fact that the fundamental number of 38 maintained itself for only a few generations and that this teratoma then changed spontaneously from 38 to 42 chromosomes shows that the more than diploid cells must have had advantages over the subdiploid cells.

A fundamental number that is two less than diploid has been maintained in some ascites tumors of the rat (*19*, *29*). But the morphologic appearance of the chromosomes in these rat tumors has shown that there has been a rearrangement of chromosome segments in the fundamental cells, and these rearrangements have presumably produced a balanced subdiploid chromosome complement.

The data on fundamental numbers in different tumors show that fundamental cells that are more than diploid can maintain themselves, the upper limit in the mouse being not less than 92 chromosomes. It can also be shown, as will be more fully discussed in a later section of this paper, that not only can more than diploid complements maintain themselves but that they may have, under certain conditions, marked advantages for survival. No subdiploid numbers have so far been maintained in the mouse, although it can be seen from the rat tumors that subdiploid fundamental cells can be maintained, presumably if accompanied by chromosome rearrangements. Since the establishment of such a range of different fundamental cells is therefore possible, chromosome numbers within this range produced in a growing tumor are potentially capable of establishing themselves as fundamental cells. The fundamental chromosome number (or numbers) is therefore the predominant chromosome number (or numbers) at a given moment, and it may be maintained as such in a tumor either for a short or long period of time.

Whereas the majority of fundamental cells presumably must undergo normal mitosis, if they are to maintain themselves, mitotic abnormalities, such as endomitosis, can be found even in cells classified as fundamental cells, and the products of these abnormalities may then establish themselves as different fundamental numbers. Even tumors started from single isolated cells can have a range of chromosome numbers (*6*, *21*, *22*),

which must have been produced by mitotic abnormalities. The observation of mitotic abnormalities in a tumor cell is, therefore, not sufficient by itself to classify the cell as a useless by-product. If abnormalities are present, abnormalities in some of the fundamental cells, in addition to abnormalities in derivative cells, can produce a constant source of genetic variation in a tumor.

A tumor is, therefore, a population of cells, some of which may have different genetic potentialities, and the potentialities of the cells that deviate from the existing fundamental cells can be determined by an experimental analysis of their subsequent behavior. It is because of these potentialities for genetic variation that tumors provide certain unique opportunities for studies in population genetics.

Chromosomes of Primary Tumors

For the comparison of nonmalignant and malignant tissues, it is necessary to use primary rather than transplanted tumors, since many changes can occur during transplantation. It has been shown in the present study that primary tumors of the mouse can be either $2n\pm$, $4n\pm$, or both $2n\pm$ and $4n\pm$. Primary tumors with and without some polyploid cells have been reported in the rat (*30*), and DNA measurements on human tumors (*31*) have shown primary tumors with and without multiple DNA classes. Primary tumors can differ in their chromosome constitution. No special pattern of chromosome numbers, however, has so far been found to be a characteristic feature of all malignant tissues.

It was originally suggested by Boveri (*32*) that the chromosomes of tumors should differ from those of nonmalignant tissues. But, in addition to the fact that no characteristic differences have been found in chromosome numbers, so far no characteristic differences in chromosome structure have been found. Certain chromosome rearrangements have been observed in some *transplanted* rat tumors (*19, 29*), but such rearrangements have not been found in the primary mouse tumors that we examined. Therefore, they are not a characteristic feature of all malignant tissues. As we have emphasized in the previous section of this discussion, more critical evidence on possible differences in chromosome structure than that obtainable in the mouse or rat can be obtained by an analysis of the chromosomes in primary tumors of the striped (Chinese) hamster or some of the marsupials, which have a low chromosome number and some very clear cytologic markers.

Chromosome Numbers and Homotransplantation

It has been found in the present study, that tumors can have, within the limits of counting errors, the same fundamental chromosome number, but, nevertheless, they can be very different in their ability to give successful homotransplants. Thus, among the tumors that originated in our C3H mice the fundamental number of $80\pm$ has been found in: sarcomas R1, R4, and R12, which have never given a successful homotrans-

plant in any of the foreign strains tested; in sarcoma R14A, which has sporadically given some successful homotransplants in some foreign strains; and in sarcomas R14B and R2A, which have given a high percentage of successful homotransplants in all foreign strains tested. Although there is a relationship between fundamental chromosome numbers and homotransplantability in that all the tumors studied in the present and other investigations (*5–7*, *25*) which are not strain specific have more than the diploid number of chromosomes, the present data clearly show, what has previously been suggested (*5*, *7*), namely, that a mere increase in chromosome number is not sufficient to produce an increase in transplantability.

Since tumors with more than the diploid number of chromosomes may or may not be strain specific, but no truly diploid tumor has been found that has lost its strain specificity, one may predict the transplantability of a diploid tumor but not the transplantability of a tumor with higher chromosome numbers. On the other hand, one may predict that a tumor that is not strain specific will have more than the diploid number of chromosomes, whereas a tumor that is strain specific may or may not be diploid.

It has also been shown that the fundamental chromosome numbers and/or the transplantability of a tumor may sometimes change during transplantation. Correlations of data on the chromosomes and on transplantability should, therefore, be based on chromosome analyses made both at the same time and after the transplantation tests. This is the procedure that we have followed in our investigations on the chromosomes and transplantability of tumors.

Immunologic Selection of Different Tumors

The immunologic selection of different tumors is clearly shown by the present data on sarcomas R14 and R2.

Sarcoma R14A, which has been continuously selected in C3H, the strain of origin, is now an 80± chromosome tumor, and it has only sporadically given some successful homotransplants. Sarcoma R14B, on the other hand, which started as the same tumor as R14A but has been continuously selected under the immunologic conditions of the originally foreign Swiss mice, is now also an 80± chromosome tumor, but it has regularly given a high percentage of successful homotransplants. Immunologic selection in Swiss mice, in contrast to selection in C3H, has produced no differences in fundamental chromosome number in sarcoma R14 within the limits of the counting errors, but there is a striking difference in transplantability.

In sarcoma R2, which also originated in C3H, continuous selection of R2B under the immunologic conditions of the originally foreign Swiss mice, in contrast to continuous selection of R2A in the strain of origin, has produced a very obvious difference in chromosome number—R2B now has a fundamental number of 45± and R2A, a fundamental number

of 80±, but there is only a very small difference between them in transplantability. Despite the striking differences in fundamental chromosome number, the differences in transplantability between R2A and R2B are very much less than those between R14A and R14B.

In R14A, where the ability to give successful homotransplants has not been continuously maintained, the cells capable of giving successful homotransplants presumably had no marked advantages over the other cells when the tumor was growing in the strain of origin. But since these cells would obviously have an advantage when the tumor was growing in a foreign strain, they would be selected out during continuous growth under the immunologic conditions in Swiss mice. The tumor would then maintain its ability to give successful homotransplants, as has, in fact, been observed in R14B. In R2A, however, the cells capable of giving successful homotransplants must have had marked advantages even in the strain of origin, since they have been maintained in the tumor in the absence of any selection for homotransplantability. Continuous growth in the foreign strain has, therefore, been able to produce a very much smaller increase in homotransplantability in R2B than in R14B.

The differences between the fundamental numbers of 80± in R2A and 45± in R2B, in addition to being reflected by a small difference in transplantability, are also reflected by differences in the rapidity with which the hosts are killed by the tumor. R2B has a shorter killing time than R2A in the foreign strain, whereas R2A has a shorter killing time than R2B in the strain of origin. In R14B, selection in the foreign Swiss, in addition to being a selection for cells capable of giving successful homotransplants, has also brought with it the selection of a shorter killing time in the strain of origin.

In both sarcomas R2 and R14, once there had been a successful transplant in Swiss mice, the ability to give successful transplants in Swiss was never lost in R2B and R14B. This was in contrast to our sarcomas R6 and R8, in which the ability to give some successful homotransplants could not be maintained by selection.

The transplantability tests in C57BR/a, BALB/c, and C57BL mice have shown that selection in Swiss has also brought with it, in R14B, the ability to give successful transplants in these three other strains of mice. Since these strains all have different genotypes, selection in Swiss produced not only a selection toward transplantability in the genotypes of Swiss mice but also a selection for transplantability in other genotypes. In sarcoma R2, both R2B, which had been selected in Swiss, and R2A, which had not been selected in a foreign strain, also gave successful transplants in C57BR/a, BALB/c, and C57BL mice. In sarcoma R2, therefore, both the presence and the absence of selection in Swiss gave successful transplants in strains with other genotypes.

Although after several transplant generations in Swiss mice both R2B and R14B gave 100 percent successful transplants in Swiss (in addition to giving successful transplants in other strains), it is also significant

that this selection in Swiss did not produce in these tumors inability to be transplanted into the strain of origin. The maintenance of this ability to be transplanted in the strain of origin has also been found in other tumors that are not strain specific. Even tumors such as sarcomas 180 and 37, that must have been selected in many different host genotypes in different laboratories, still show a preference for certain host strains, which may be the strains with genotypes similar to the mice in which these tumors originated (*12*, *33*).

Tumors that are not strain specific are different from tumors such as the 6C3HED ascites, selected by growing in Swiss mice (*6*), since in this case the selected tumor seems to be losing its ability to be transplanted into the strain of origin in addition to being still unable to kill in Swiss mice. They are also different from the tumors selected during growth in F_1 hybrids (*21*, *34–37*), which are changed in their transplantability into F_2 or backcross hybrids, but not in their transplantability into foreign strains. There may also be a further difference between the selection for loss of strain specificity and the selection in F_1 hybrids, since the selection in F_1 hybrids is fully effective after a single passage in the F_1 host, whereas the complete selection for loss of strain specificity was produced in sarcomas R2 and R14 only after several transplant generations. In our previous studies on ascites tumors (*7*), there was also no apparent selection of different chromosome numbers in ascites tumors grown for a single generation in a foreign host. A selection of hypertetraploid cells has, however, been obtained from the hyperdiploid Ehrlich ascites tumor (*26*) after one passage in a host which had previously been immunized against the hyperdiploid Ehrlich by the tail-inoculation method (*38*).

The selection of hypertetraploid tumors from the hyperdiploid Ehrlich is of further interest in that the hyperdiploid Ehrlich can be successfully transplanted into different strains of mice after intraperitoneal transplantation (*25*), and tests on the Landschütz I tumor, which seems to be the same tumor as the hyperdiploid Ehrlich (*23*), have shown 100 percent transplantability into all strains of mice tested (C3H, C57BR/a, BALB/c, C57BL, and Swiss) not only after intraperitoneal but also after intramuscular and subcutaneous transplantation (Sachs, unpublished data). Although it is not yet known in which other characteristics beside chromosome number the derived hypertetraploids differ from the hyperdiploid tumor, the fact that hypertetraploid tumors could be obtained by immunologic selection shows that such selection is possible even in the hyperdiploid Ehrlich with its high degree of homotransplantability.

The results of selection on sarcoma R14 show that it is possible to obtain immunologic selection in a tumor which, in the absence of such selection, does not maintain its ability to give successful homotransplants. The results of selection on sarcoma R2, which are supported by the immunologic selection of hypertetraploid tumors from the hyperdiploid Ehrlich, show that it is also possible to obtain immunologic selection in a tumor which, in the absence of such selection, can maintain its ability to give a high degree of homotransplantability.

Histocompatibility Genes

There is extensive evidence (reviewed in *39–44*) that strain-specific tumors and nonmalignant tumors require for successful transplantation certain genetic similarities in the host and in the transplanted tissue. The genes concerned in the success of this transplantation are called histocompatibility (*H*) genes. It is known that in the mouse (*41, 43, 45*) *H* genes exist at different genetic loci and that the *H-2* locus, which has been most intensively studied, exists as a series of alleles. (These alleles are really pseudoalleles, as will be shown below.)

According to the classical theory of transplantation, one allele at each *H* locus was supposed to be recessive, but an analysis of the *H-2* locus has shown that not one of its alleles behaves as a recessive (*43, 45*). With respect to the absence of recessiveness, therefore (*43, 45*), the *H-2* genes behave in a way similar to certain blood-group genes in man (*46*), cattle (*47*), and chickens (*48*). In accordance with other systems, the different alleles (pseudoalleles) at an *H* locus and the different *H* loci should therefore be designated not by a small but by a capital letter, and we have adopted this procedure, in conformity with that suggested for blood groups (*49*).

It is known (*43, 44*) that, although a tumor originating in, *e.g.*, either an $H\text{-}2^D/H\text{-}2^D$ strain or an $H\text{-}2^K/H\text{-}2^K$ strain can be successfully transplanted into an F_1 hybrid $H\text{-}2^D/H\text{-}2^K$, a tumor originating in the F_1 hybrid cannot be successfully transplanted into either of the parental strains. It is of interest that these facts in themselves do not agree with the idea of dominance and recessiveness at *H* loci, since on the basis of dominance the unsuccessful transplantation of the F_1 hybrid tumor $H\text{-}2^D/H\text{-}2^K$ into an $H\text{-}2^D/H\text{-}2^D$ host would imply the dominance of $H\text{-}2^K$, and the unsuccessful transplantation of the same tumor into an $H\text{-}2^K/H\text{-}2^K$ host would imply the dominance of $H\text{-}2^D$.

That each *H* allele may act independently, even in the host, can be seen from the changes produced in tumors grown for a single generation in an F_1 hybrid (*21, 34–37*). Such tumors are sometimes more and sometimes less transplantable into F_2 and backcross hybrids. The *H* allele in the F_1 hybrid, which differs from that of the tumor, thus seems to be immunologically effective in that it can cause the selection, from the mixed population in a tumor, of cells with different degrees of resistance to the immunologic responses that prevent a successful transplant in the F_2 and backcross hybrids.

In addition to the absence of recessiveness, it can be seen from the published data that the *H-2* locus shows a dosage effect and pseudoallelism. (The discussion in this section does not, of course, include the behavior of tumors that have lost their strain specificity.)

Dosage effect.—Evidence for a difference in immunologic response toward a single, as against a double, dose of an *H* antigen can be seen in the observation that, in the mouse, the erythrocyte antigens controlled by *H* genes have a weaker immunologic reaction in the heterozygous F_1 hybrid than in the homozygous parents (*50*). This is in agreement with

a similar phenomenon in certain blood groups in some other species (*46*, *51*, *52*).

Pseudoallelism.—The A strain of mice (*45*, *53*) has the constitution $H\text{-}2^{DK}/H\text{-}2^{DK}$, and this strain has a common origin (*54*) with strains that are $H\text{-}2^{D}/H\text{-}2^{D}$ or $H\text{-}2^{K}/H\text{-}2^{K}$. The fact that there are mice with $H\text{-}2^{D}$ and $H\text{-}2^{K}$ on the same chromosome and mice with $H\text{-}2^{D}$ and $H\text{-}2^{K}$ on different chromosomes shows that crossing over must have occurred between $H\text{-}2^{D}$ and $H\text{-}2^{K}$. Strictly speaking, the *H-2* alleles are, therefore, pseudoalleles (*55*, *56*), and a search may reveal the existence of other cross-over types.

Chromosome Duplication and Loss of Strain Specificity

Although there are tumors that are clearly strain specific and those that are clearly not strain specific, tumors belonging to the latter category do not all have the same degree of homotransplantability. Thus, of the three ascites tumors that we have studied originating in C3H mice (*7*, *57*), the 6C3HED tumor is strictly strain specific and has given no successful homotransplants, even when transplanted intraperitoneally in C57BR/a, BALB/c, C57BL, or Swiss mice (it does not even give 100% successful transplants when transplanted subcutaneously into our C3H mice); the MC1M tumor has given 100 percent successful homotransplants in these strains of mice when transplanted intraperitoneally but none when transplanted intramuscularly or subcutaneously; and the MC1A tumor has given 100 percent successful homotransplants in these strains of mice when transplanted intraperitoneally or intramuscularly but a lower percentage when transplanted subcutaneously. Both MC1M and MC1A have maintained their ability to give 100 percent successful transplants in the strain of origin at all three transplantation sites. The Krebs-2 and Ehrlich ascites tumors also give some unsuccessful transplants in some hosts when transplanted subcutaneously (Sachs, unpublished data), and some unsuccessful transplants have been observed with tumors such as sarcoma 180 and sarcoma 37 (*12*, *33*). Tumors can therefore have different degrees of loss of strain specificity, and there have invariably been some unsuccessful transplants among all tumors tested in a sufficient number of host strains.

The data on fundamental chromosome numbers and strain specificity obtained in the present investigation, which are summarized in table 6 for the tumors that originated in our C3H mice, have substantiated the previous conclusion (*7*) of a relationship between chromosome duplication and homotransplantability. They also show that duplication, which would include duplication of *H* genes that can show a dosage effect, has not by itself produced a loss of strain specificity. It has not been possible so far to carry out an experiment which is the opposite of duplication in the tumor, *i.e.*, duplication of the chromosomes of the host. This is due to the fact that, although it can be shown that such duplication has taken place during the course of evolution (*14*), a polyploid animal has not yet been experimentally produced in mammals (*58*). Such an

TABLE 6.—*Fundamental chromosome numbers and homotransplantability of the solid tumors that originated in our C3H mice—detailed data are given in tables 2 and 3 and in the text*

Chromosome numbers of tumors originating in C3H ($H\text{-}2^K$) mice	Homotransplantation − = unsuccessful homotransplants + = successful homotransplants			
	C57BR/a mice ($H\text{-}2^K$)	BALB/c mice ($H\text{-}2^D$)	C57BL mice ($H\text{-}2^B$)	Swiss mice ($H\text{-}2$?)
40±	−	−	−	−
40± and 80±	−	−	−	−
80±	−	−	−	−
45±	+	+	+	+
80±	+	+	+	+

experiment, however, could be performed in some amphibians, where many polyploid animals have been experimentally produced (*59*).

In the hyperdiploid Ehrlich ascites tumor it has been possible to obtain immunologic selection of hypertetraploid cells from a predominantly hyperdiploid tumor (*26*). But the hyperdiploid Ehrlich itself has a high degree of homotransplantability, so that the difference between those two types of cells, one of which seems merely to contain a complete duplication of the chromosome set of the other, is not associated with a difference between strain specificity and the loss of strain specificity.

The data on the chromosomes and strain specificity also show that successful homotransplantability does not require the duplication of all the chromosomes. Both our sarcoma R2B and the hyperdiploid Ehrlich (Landschütz) ascites have a fundamental number of 45± chromosomes, and this is the lowest number found so far in tumors with a high degree of homotransplantability. Duplication of only some, presumably special, genetic material seems to be necessary in order to produce successful homotransplantability. Whether duplication of about five chromosomes or chromosome segments or duplication of a lower number is the minimum necessary could be determined by finding tumors with fundamental numbers of less than 45± that are not strain specific.

Duplication by itself has not produced a loss of strain specificity, and this also seems to be true of mutation. For example, a loss of strain specificity cannot be due to mutation from one pair of *H* pseudoalleles to another, since this would merely make the tumor specific for hosts with the new *H* pseudoallele and would prevent the tumor from being successfully transplanted into the strain of origin. The mere mutation of *H* genes to inactivation, if this is possible (and the same argument would apply to mutations of other genes), also seems to be ruled out as an explanation in view of the relationship between chromosome duplication and homotransplantability. If such inactivation mutations are to produce a loss of strain specificity, a tumor with duplicate chromosome material would require mutations at more genetic loci than would a strictly diploid tumor. One should find among tumors that are not strain specific a high proportion of diploids, whereas the experimental findings have

shown no diploid tumor at all. In the same way, the loss of chromosomes or chromosome segments carrying the *H* genes is by itself not a sufficient explanation, since tumors with chromosome numbers higher than diploid would be at a disadvantage in that they would have to lose more chromosome material than diploid tumors. The possibility that the loss of such material in a diploid tumor might produce inviable subdiploid cells would not invalidate this point, because a tumor with higher chromosome numbers would have the same disadvantage, since it is unlikely that a loss of chromosome material with *H* genes could be compensated by the presence of other chromosome material in duplicate.

The loss of strain specificity, therefore, does not seem to be explainable merely by loss of antigenicity, and, although this certainly does not necessarily mean that such a process cannot exist, there has been no evidence so far that a loss of antigenicity actually has taken place. On the other hand, the fact that all tumors tested show some unsuccessful transplants in some hosts and that it has been possible to obtain immunologic selection from tumors such as sarcoma R2A and the hyperdiploid Ehrlich ascites, which have a high degree of homotransplantability, shows the antigenicity of these tumors.

We have obtained evidence (*57*) that tumors with different degrees of homotransplantability have different degrees of resistance to the immunizing responses of the host. Therefore, a loss of strain specificity seems to be correlated not with a loss of ability to immunize, but with an increased resistance to the immunization.[4] The mechanism of this resistance is being further investigated. Although the antibodies against erythrocytes do not seem to be those that prevent the growth of a transplant, it is relevant to the present point that high titers of such antibodies may be present in a foreign host that is being killed by the growth of a tumor (*60–62*).

It can be concluded on the basis of the available evidence that both strain-specific tumors and those that are not can produce immunizing responses and that the success of a homotransplantation is dependent on whether the immunologic responses of the host can kill the tumor rather than the reverse. That these two possibilities do exist, even in the same host-tumor combination, can be seen in our data on the MC1M ascites (*7*, *57*), which gives 100 percent successful homotransplants when transplanted intraperitoneally, but none when transplanted intramuscularly. This difference according to transplantation site seems to be due to the fact that after intraperitoneal transplantation the tumor can kill the host before the host can kill the tumor, whereas after intramuscular transplantation the opposite is true. In sarcoma R14B, selection in Swiss has given successful homotransplants not only in Swiss mice but also in mice belonging to other strains, and this would show that a gain in resistance to the immunologic responses of one genotype can bring with it a gain in resistance to the immunologic responses of other genotypes.

[4] This fact is also of importance in evaluating the results of therapeutic studies based on the use of transplantable tumors that are not strain specific.

While neither duplication nor mutation by themselves can explain the loss of strain specificity, this genetic change in the behavior of a tumor can be explained by the occurrence of both these processes together. Presumably it is special genetic material that must be duplicated, so that the right sort of duplication would be required for a loss of strain specificity. It can also be presumed that the right sort of mutation would be required. The existence of different degrees of homotransplantability can then be explained as follows: If duplication of more than one genetic locus is involved, there can be different amounts of the right duplications, and the same is true with respect to mutations. Thus, the 65± chromosome MC1M ascites may contain less of the right duplicate material than the 45± chromosome hyperdiploid Ehrlich (Landschütz) ascites or sarcoma R2B, and this could account for the fact that the MC1M ascites has a lower degree of homotransplantability than these other two tumors.

If the mutation or mutations necessary are nuclear, either in the form of nuclear-gene mutations or positional effects, it is more likely that they would occur before duplication, because, if they occurred after duplication, mutation at more loci would be required. Thus it may be significant that both sarcomas R2 and R14 began as 2n± and 4n± tumors, and we are investigating this point further. If, however, the mutation or mutations are cytoplasmic, since it is known that there is duplication of nuclear genetic material but not if this also includes duplication of cytoplasmic genetic material, it would be more difficult to decide whether the mutation(s) would tend to precede or follow the duplication(s). The type and number of mutations necessary, which may also be both cytoplasmic and nuclear, may eventually be determined when the mechanism of resistance, found in tumors that are not strain specific, has been clarified. It will then also be possible to show the respective roles of duplication and mutation in determining the ability of a tumor to give successful homotransplants.

Summary

The chromosomes and strain specificity in successive transplant generations have been studied in 12 solid tumors of the mouse. The studies were begun as soon as possible after the time of tumor induction.

In 2 tumors that originated in C3H mice, sarcomas R2 and R14, some successful homotransplants were obtained in Swiss mice the second time the tumor was transplanted. Two sublines, called R2B and R14B, were selected by continuous growth in Swiss mice, and these were in both cases genetically different from R2A and R14A, the sublines that were continuously grown in the strain of origin.

Sarcoma R14 began with diploid ± and tetraploid ± fundamental chromosome numbers. After 16 transplant generations, both R14A and R14B now have a fundamental number of 80±, and, whereas R14B has regularly given a high percentage of successful homotransplants in all strains of mice tested, R14A has given successful homotransplants only sporadically in some strains of mice. In R14B, therefore, selection in Swiss

has brought with it the ability to give successful homotransplants not only in Swiss mice but also in strains of mice with other genotypes.

Sarcoma R2 also began with diploid ± and tetraploid ± fundamental numbers. After 14 transplant generations, R2A now has a fundamental number of 80±, R2B now has a fundamental number of 45±, and both R2A and R2B have regularly given a high percentage of successful homotransplants in all strains of mice tested.

Despite continuous selection in a foreign genotype, R2B and R14B have fully maintained their ability to be successfully transplanted into the strain of origin. R14B killed more rapidly than R14A in the strain of origin. On the other hand, R2B killed more slowly than R2A in the strain of origin and more rapidly than R2A in Swiss mice.

The other 10 tumors studied originated either in C3H or in Swiss. Two carcinomas and 6 sarcomas gave no successful homotransplants in any of the strains of mice tested, and 2 sarcomas showed a temporary loss of strain specificity. These 10 tumors were examined for periods ranging from 5 to 18 transplant generations, and those that maintained their strain specificity had fundamental numbers that were either 40±, 80±, or both 40± and 80±. The differences in fundamental number in these tumors were not related to the differences in the mean survival times of the hosts.

One sarcoma with fundamental numbers of 40± and 80± and 1 sarcoma with a fundamental number of 80± gave only a few successful homotransplants. Unlike either sarcoma R14, where a permanent loss of strain specificity could be produced by immunologic selection in a foreign genotype, or sarcoma R2, where there was a permanent loss of strain specificity both in the presence and the absence of such selection, in these two tumors, the ability to give successful homotransplants was neither maintained during growth in the strain of origin nor could it be selected out by growth in a foreign genotype.

A study of 20 primary tumors of the mouse has shown that primary tumors can have diploid ± , tetraploid ± , or both diploid ± and tetraploid ± fundamental numbers. The chromosome studies during successive transplant generations have shown in some tumors, changes in fundamental number during transplantation.

The present correlation of chromosome studies and transplantability tests has substantiated the previous conclusion on the relationship between chromosome duplication and homotransplantability. All tumors studied that can give successful homotransplants have more than the diploid number of chromsomes, whereas tumors that are strictly strain specific may or may not be more than diploid. The existence of 80± chromosome tumors that are strain specific has shown that duplication by itself is not sufficient to produce a loss of strain specificity, and the existence of 45± chromosome tumors with a high degree of homotransplantability has shown that not all chromosomes must be duplicated.

A loss of strain specificity, therefore, does not seem to involve the actual loss of genetic material but, on the contrary, a gain of genetic loci. The

ability to give successful homotransplants seems to be due not to a loss of the ability to immunize but to a gain in resistance to the immunizing responses of the host.

It can be concluded from the available evidence that mutation by itself would not produce homotransplantability. While the loss of strain specificity, therefore, cannot be explained either by duplication or by mutation alone, the genetic basis of successful homotransplantation, including the existence of various degrees of homotransplantability, can be explained by the existence of both these processes.

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PLATE 69

Photomicrographs of the chromosomes of solid tumors of mice, × 2,000. Feulgen squashes. Figures 1, 2, 3, 5, and 6, pretreatment with hypotonic salt solution.

FIGURE 1.—Sarcoma R19, transplant generation 5. Cell with 40± chromosomes.

FIGURE 2.—Sarcoma R14B, transplant generation 2. Cell with 40± chromosomes.

FIGURE 3.—Sarcoma R14B, transplant generation 8. Cell with 80± chromosomes.

FIGURE 4.—Sarcoma R8, transplant generation 1. Cell with 55± chromosomes.

FIGURE 5.—Sarcoma R6, transplant generation 15. Cell with 80± chromosomes.

FIGURE 6.—Sarcoma R12, transplant generation 15. Cell with 80± chromosomes.

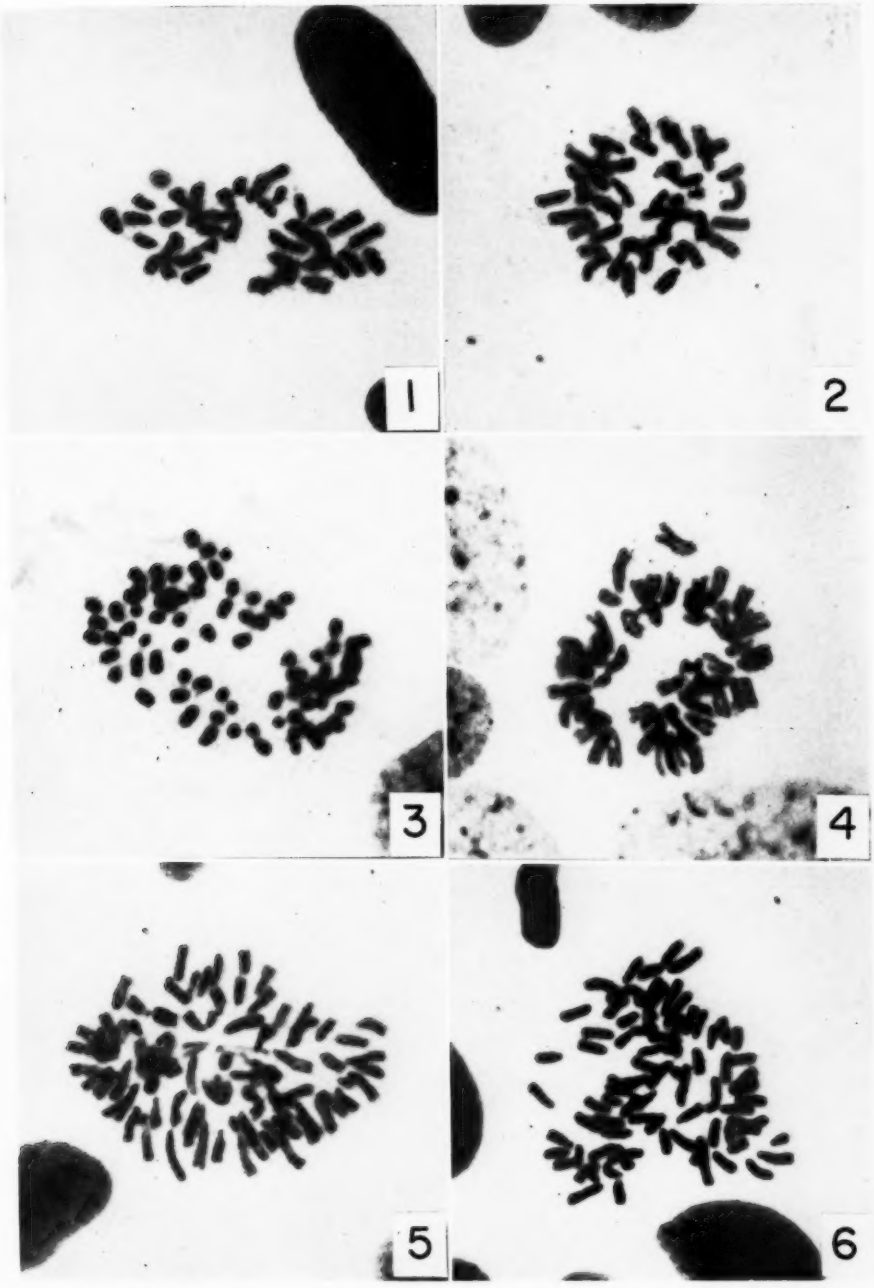

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PLATE 70

Photomicrographs of the chromosomes of solid tumors of mice, × 2,000. Feulgen squashes. Figures 7, 8, 9, and 11, pretreatment with hypotonic salt solution.

FIGURE 7.—Carcinoma W2, transplant generation 4. Cell with 40± chromosomes.

FIGURE 8.—Carcinoma W1, transplant generation 17. Cell with 40± chromosomes.

FIGURE 9.—Sarcoma R12, transplant generation 9. Cell with 80± chromosomes.

FIGURE 10.—Sarcoma R8, transplant generation 1. Cell with 80± chromosomes.

FIGURE 11.—Sarcoma R12, transplant generation 5. Cell with >80 chromosomes.

FIGURE 12.—Sarcoma R8, transplant generation 2. Cell with several hundred chromosomes and multipolar spindle.

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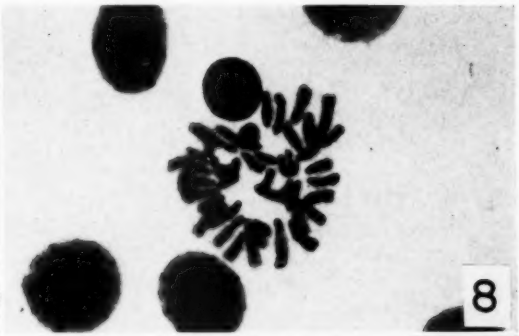
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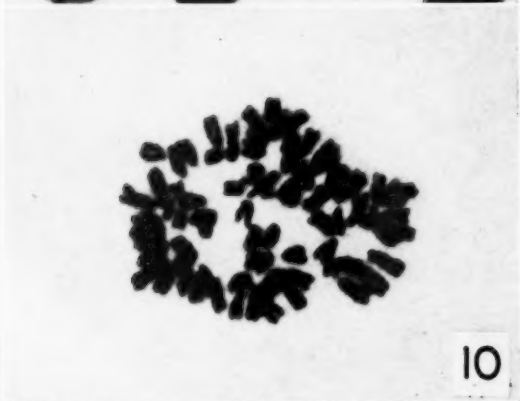
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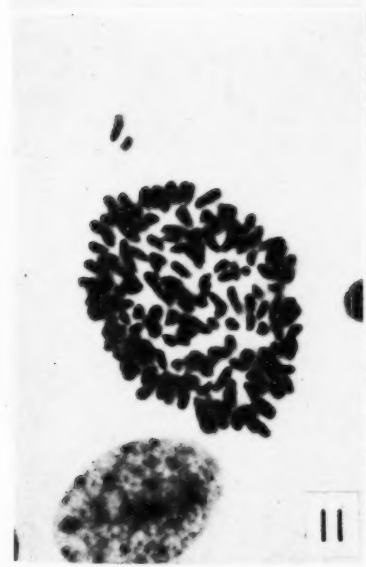
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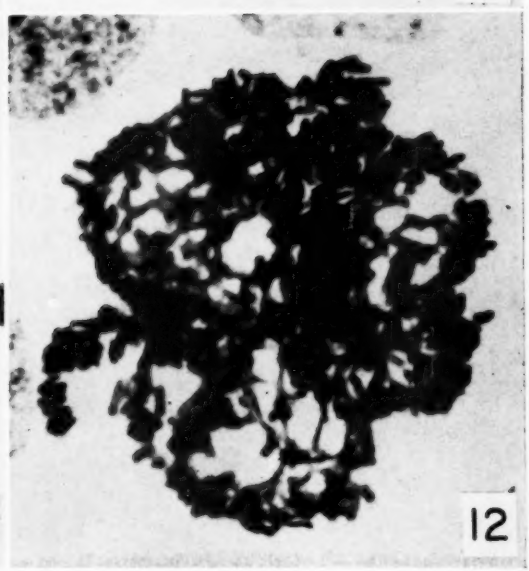
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PLATE 71

Photomicrographs of histologic sections of solid tumors of mice. Hematoxylin and eosin. × 360

FIGURE 13.—Carcinoma W1, transplant generation 12.

FIGURE 14.—Sarcoma R4, transplant generation 11.

FIGURE 15.—Sarcoma R6, transplant generation 12.

FIGURE 16.—Sarcoma R12, transplant generation 8.

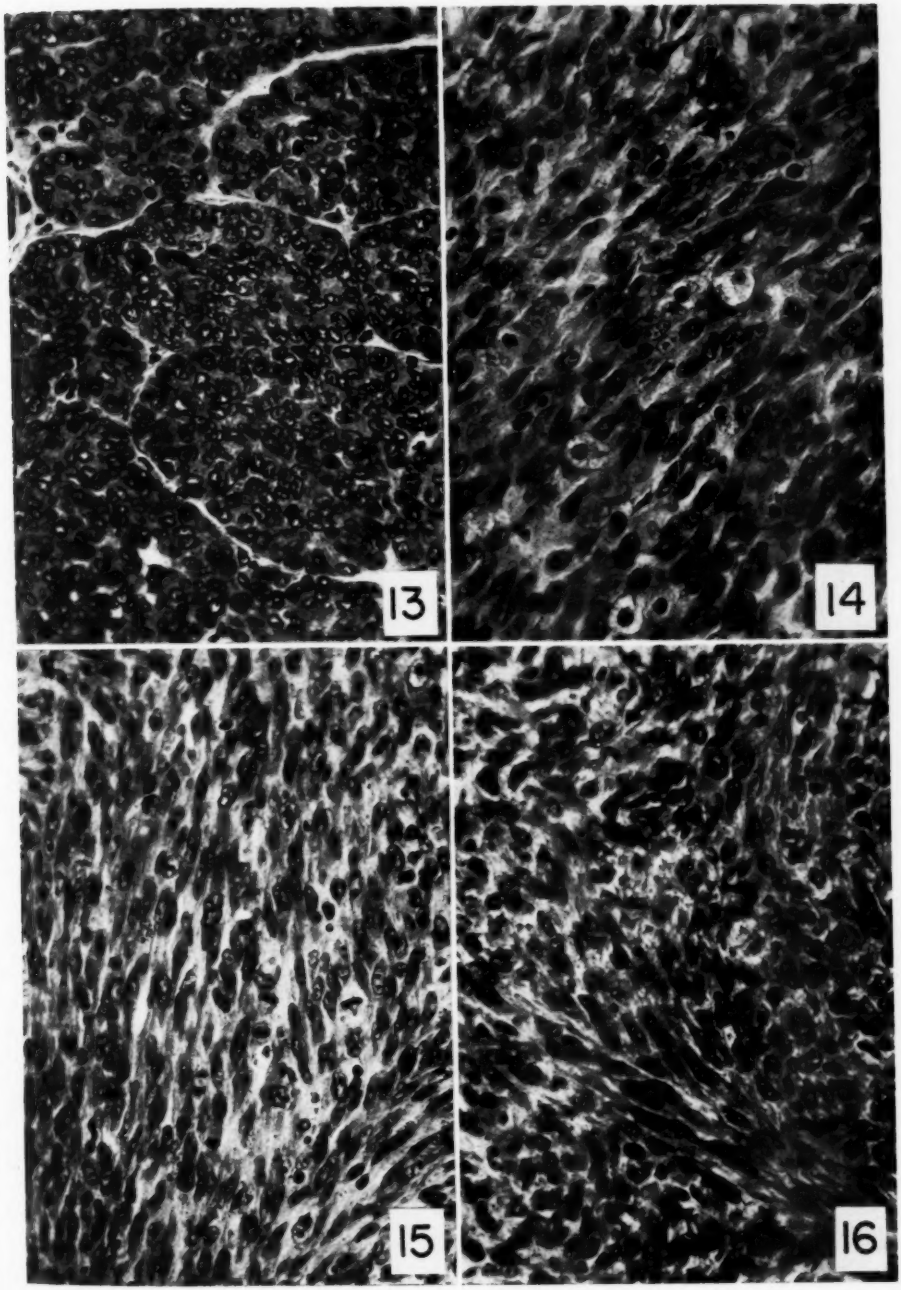
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PLATE 72

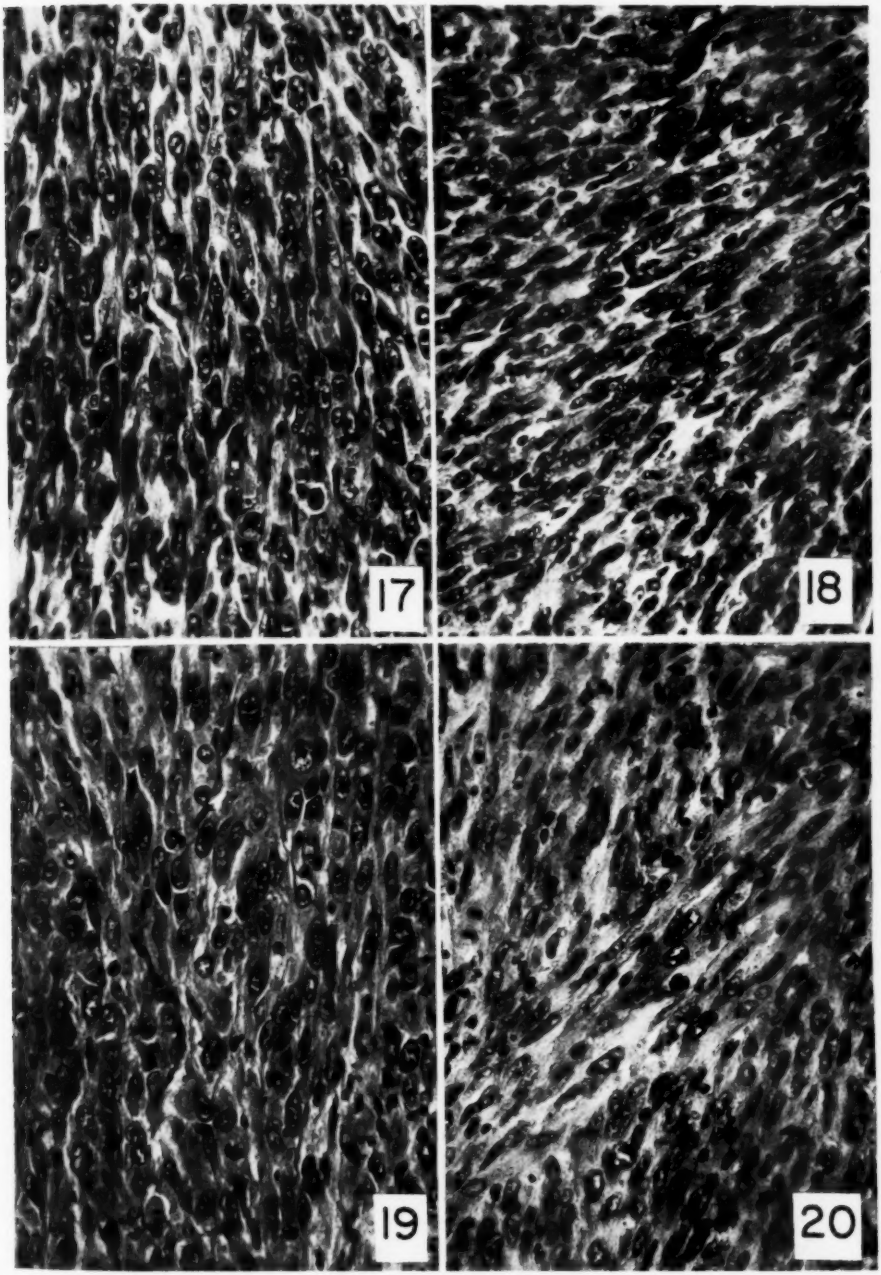
Photomicrographs of histologic sections of solid tumors of mice. Hematoxylin and eosin. × 360

FIGURE 17.—Sarcoma R14A, transplant generation 11.

FIGURE 18.—Sarcoma R2A, transplant generation 5.

FIGURE 19.—Sarcoma R14B, transplant generation 11.

FIGURE 20.—Sarcoma R2B, transplant generation 5.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Biological Studies on the Rous Sarcoma Virus. IV. Interpretation of Tumor-Response Data Involving One Inoculation Site per Chicken[1]

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Introduction

The sources of technical and biological variations encountered in quantitative biological experimentation with the Rous sarcoma virus, as well as methods that have been developed for their control, were reviewed and discussed in a preceding report of this series (*1*). Among the problems needing further study is that of the control of test-animal variations in bioassay experiments involving only one inoculation site on each chicken. Multiple-site designs [*cf.* (*1*)], although fairly efficient for the control of variations due to differences between chickens, are limited in their practical applicability because of the narrowness of the potency span that can be tolerated on a given chicken without disturbance of the balanced design caused by certain complicating factors [*cf.* (*1*)].

Using single-site inoculations in chickens of inbred lines, Carr and Harris (*2*) and Kenzy (*3*) reported successful assays of this virus by means of the tumor-incidence response and the serial-dilution method commonly employed for the assay of other animal viruses [*cf.* (*4*)]. No mention was made in either instance of heterogeneity which might complicate interpretations of the tumor-incidence responses. The biological results in their chicken populations, therefore, may have been homogeneous. This is not the case with the chickens from a commercial source used as test animals in this laboratory. Although the results are homogeneous in many lots of chickens, in others an occasional chicken may deviate widely from the remaining individuals of the same test group in its biological reaction to the virus. Aberrant individuals may develop tumors only after excessively prolonged latent periods, or, may be refractory to even the strongest doses of virus.

A similar type of heterogeneity was observed by Eckert, Beard, and Beard (*5*) [see also (*1*) and (*4*)] in the responses to another fowl tumor virus (that of erythromyeloblastic[3] leukosis), even when the chickens

[1] Received for publication July 5, 1955.

[2] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

[3] It has been concluded in a recent report by Eckert *et al.* (*6*) that the disease investigated in the laboratories at Duke University is a relatively pure strain of myeloblastosis. As a consequence, the properties attributed to the virus designated by the general term "erythromyeloblastosis" are now regarded specifically as those of the agent of avian myeloblastic leukosis.

366462—56——5

were from inbred lines. These investigators also demonstrated that the heterogeneity of their latent-period data caused by the aberrant chicken responses could be controlled statistically by treating their time-frequency curves as truncated normal distributions and applying methods of analysis appropriate to such distributions, which have been described in detail by Bliss (*7*) and Ipsen (*8*). The same methods are also applicable to the analysis of heterogeneous latent-period data obtained with single-site inoculations of the Rous sarcoma virus.

The biological responses to certain viruses, particularly those associated with neoplasia, differ in variability from the responses of highly susceptible hosts to some of the more virulent viruses of infectious diseases. As a result, special methods have had to be employed for their analysis and interpretation [*cf.* (*1*, *4*, *5*, *9–14*)]. The analytical methods found to be most suitable are adaptations of procedures that were developed in the fields of pharmacology and biological standardization [see (*15*) for references to general descriptions of bioassay methods as applied in these fields].

The purpose of the present report is to show typical data of both the homogeneous and heterogeneous types obtained with the Rous sarcoma virus, and to illustrate, by graphic methods, the principles that have been applied to analyses of both the latent-period and tumor-incidence types of responses resulting from single-site inoculations on each chicken. The illustrations include for both homogeneous and heterogenous types of data: (*a*) Time-frequency curves representing the distributions of latent-period responses within dose groups; (*b*) the curve relating mean latent-period response and log dose; and (*c*) the curve relating tumor-incidence response and log dose. The further use of such curves as research tools for quantitative biological investigations (*e.g.*, in bioassays, in comparisons of host reactions, and in studies on the kinetics of virus-host interactions) is beyond the scope of the present communication, which deals only with the basic distributions from which they are derived. Examples of the use of dose-response curves in parallel-line assays of the Rous sarcoma virus have been given previously (*16*), as well as in an accompanying publication (*17*). Another accompanying report (*18*) illustrates the use of time-frequency curves for comparing host reactions among different experimental groups in which all test animals received the same dose of virus. A review has also been given previously (*1*) of the principles of potency estimation that have been applied to the measurement of biological activity of viruses, including that which causes the Rous sarcoma.

Materials and Methods

Test chickens.—The test chickens were of the New Hampshire Red breed. They were derived from a constant commercial source which maintains its own breeding flock. All chickens contributing to the data reported herein were 23 days old at the time of inoculation with virus.

Standard virus.—A single batch of standard virus, frozen lot CT 581 [*cf.* (*14*)], was used for the experiments reported herein. The methods of

thawing and diluting were identical with those described in a previous report (*14*). The diluent was 0.05 *M* citrate buffer *p*H 6.7, containing 1 mg. per 100 ml. of hyaluronidase (*14*).

Inoculation of chickens.—The site of inoculation on each chicken was the subcutaneous tissue of the left wing web. The site location was standardized because of an unexplained systematic difference between responses at comparable sites on the opposite wings [see also (*18*)]. The inoculating needle (20-gauge) was inserted through the skin over the muscles on the ventral surface of the forewing and passed through the muscle tissue to the point where the ventral and dorsal skin layers become closely approximated to form the wing web. The inoculum was delivered at this point, forming a "blister" seated at the edge of the muscle and distending the ventral skin layer of the wing web. The muscle tissue acted as a valve to prevent back leakage of the inoculum through the needle track. The volume (0.2 ml.) of the inoculum was constant for all injections.

Examination of chickens.—Beginning on the 5th day following inoculation, each chicken was examined daily for the next 5 days (*i.e.*, through the 10th day following inoculation) and at progressively increasing time intervals thereafter according to the schedules shown in the accompanying tables [see also reference (*13*)]. The examination schedule was arranged, insofar as possible, to provide equal intervals on a reciprocal-of-time scale, since the metameter found to be most desirable for analyses of the latent-period data was the reciprocal, in days, multiplied by 100 to avoid decimal fractional values (*i.e.*, $y=\frac{100}{\text{days}}$) (*12*, *14*). It is not practical, however, to reduce the examination interval to fractions of a day (*13*), and the lower limiting interval of 1 day was used for the first few days of the observation period. Early tissue reactions were readily detectable by sight in the translucent wing webs, and when the neoplastic nature of a lesion was confirmed by progressive growth [*cf.* (*12*)], the tumor was considered as having appeared at the midpoint of the examination interval during which its presence was first recorded (*13*).

Experimental Results

Two sets of experimental results involving adequate numbers of chickens in each dose group were selected for illustrating the different types of distributions of individual chicken responses that have been encountered in work with the chicken population employed in this laboratory (commercial source, see preceding section). Both were obtained with the same standard preparation of virus (CT 581) in chickens of the same age, but at different times. They therefore involved different hatching lots of chickens.

The first set of data (A) represents part of an experiment (CT 593) initiated on January 14, 1954, in which other standard virus preparations were also tested at the same time in comparable chicken groups. The primary data obtained with frozen material CT 581 in this experiment are given in detail in table 1. As will be shown in the following section, these data are homogeneous and are adequately represented by normal

TABLE 1.—*Detailed responses of type A (homogeneous); see text*

Examination interval					Frequencies (f) of chickens developing tumors at successive times and dilutions								
Days		$\frac{100}{\text{days}}$			Dilution of virus								
Last neg.	First pos.	Last neg.	First pos.	Midpoint of interval (y)	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}
4	5	25.0	20.0	22.5	16*	17	7						*
5	6	20.0	16.7	18.3	1	3	8	10				*	
6	7	16.7	14.3	15.5	*		2	8	5				
7	8	14.3	12.5	13.4			3	2	13	4			
8	9	12.5	11.1	11.8	1				1	6	2		
9	11	11.1	9.1	10.1					1	4	1		
11	13	9.1	7.7	8.4						3	1		
13	15	7.7	6.7	7.2							1		
15	20	6.7	5.0	5.8									
Sum positive (n_+)					18	20	20	20	20	17	5	0	0
No. originally inoculated (n_o)					20	20	20	20	20	20	20	20	20
No. dying neg. during expt. (n_d)					2	—	—	—	—	—	—	1	1
Corrected total no. ($n=n_o-n_d$)					18	20	20	20	20	20	20	19	19

*= Death of one animal without tumor within interval indicated.

distributions, both with respect to the tumor-incidence response and to the latent-period response in terms of appropriate metameter units.

The second set of data (B), shown in table 2, represents an experiment (CT 584) initiated on September 23, 1953, in which 5 separate samples of the same standard virus (lot CT 581) were tested simultaneously in random groups of chickens drawn from a common hatching lot. Analyses of the results obtained with the separate samples have already been published (*14*). Since no significant differences were observed (*14*) between the results of separately frozen samples, the data for all samples have been combined to provide the larger numbers of observations within the dose groups presented in table 2. These data illustrate the type of distribution in which some of the individual responses deviate markedly from those of the remainder of the same group, and for which no normalizing metameter could be found which would include all of the individual responses.

Interpretation of Tumor-Response Data

Latent-Period Responses

Data within Dose Groups

Homogeneous chicken responses (*data of type A, table 1*).—The frequencies of chickens developing tumors within successive time intervals are shown for each separate dose in table 1. The nature of the distribution of individual chicken responses, as well as the *mean* and the *standard deviation*, may be determined separately for each dose group by procedures (graphic or algebraic) described by Bliss (*7*) for time-frequency data of this type. As illustrated for one of the dose groups (dilution 10^{-5}) in table 3, the frequencies are accumulated with time, and the cumulative values, in percent, are plotted directly on probability paper,[4] or they are converted to probits (*19–21*) for plotting on ordinary graph paper against the *upper class-limit* values,[5] as illustrated in text-figure 1. A satisfactory fit of the plotted points to a straight line indicates that, for the practical purposes of bioassay, the responses within dose groups may be considered as being normally distributed in terms of the time metameter employed (*7*). The plotted points of text-figure 1 appeared to be consistent with a linear relationship, and the straight line was drawn through them by sight. Where the points are more scattered, or where they appear to deviate systematically from a straight line, however, it may be necessary to use more animals or to apply statistical tests to determine whether they are consistent with a linear relationship (and therefore with a normal distribution). Such tests have been described in detail by Bliss (*7*).

The data of all dose groups of table 1 within the significant dose range (10^{-3} through 10^{-6}) are plotted conjointly in text-figure 2. The results

[4] A suitable type of probability paper is that designed by Dr. J. Berkson and printed under the designation "No. 32,451. Normal Ruling" by the Codex Book Co., Inc., Norwood, Mass.

[5] The time metameter in units of $\frac{100}{\text{days}}$ is employed in reverse order in the present analyses to facilitate comparison with the original time intervals (in days) actually used in recording the observations (see tables 1 and 2). However, the original units may be dropped altogether and the analyses carried out with the reciprocal (metameter) values in their natural order, if so desired.

TABLE 2.—*Detailed responses of*

Examination interval					Frequencies (f) of chickens			
Days		$\frac{100}{\text{days}}$			Dil. 10^{-3}		Dil. 10^{-3}	
Last neg.	First pos.	Last neg.	First pos.	Midpoint of interval (y)	Sample a b c d e	sum	Sample a b c d e	sum
4.....	5	25.0.....	20.0	22.5	6 7 7 7 5	32	2 1 3 3 2	11
5.....	6	20.0.....	16.7	18.3	1 1 3 1 5	11	5 7 4 5 7	28
6.....	7	16.7.....	14.3	15.5	2 1 2	5	2 1 2 1	6
7.....	8	14.3.....	12.5	13.4	1 1	2	1	1
8.....	9	12.5.....	11.1	11.8			1	1
9.....	10	11.1.....	10.0	10.6			1	1
10.....	12	10.0.....	8.3	9.2			1	1
12.....	14	8.3.....	7.1	7.7				
14.....	16	7.1.....	6.3	6.7				
16.....	20	6.3.....	5.0	5.6				
20.....	27	5.0.....	3.7	4.4				
Sum positive (n_+)						50		49
No. originally inoculated (n_o)						50		50
No. dying neg. during expt. (n_d)						—		—
Corrected total no. ($n=n_o-n_d$)						50		50

*Dilution 10^{-4} was also tested, but it produced no tumors in 50 chickens. One chicken inoculated with sample c of this dilution died between the 16th and 20th days.

†Death of one animal without tumor within interval indicated.

for all groups are seen to be consistent with linear relationships. It may be concluded, therefore, that the individual responses are normally distributed within dose groups in terms of the response metameter $\left(y=\frac{100}{\text{days}}\right)$

TABLE 3.—*Illustrating the derivation of cumulative percentage frequencies and their probit equivalents from time-frequency data within doses—data of dose group 10^{-5} of table 1*

Examination interval				Frequency (no. of chickens with tumors)	Cumulative frequency		
Days		$\frac{100}{\text{days}}$					
Lower limit (last neg.)	Upper limit (first pos.)	Lower limit* (last neg.)	Upper limit* (first pos.)	(f)	Number	Percent	Probit†
5.....	6	20.0.....	16.7	0	0	0	—
6.....	7	16.7.....	14.3	5	5	25	4.325
7.....	8	14.3.....	12.5	13	18	90	6.282
8.....	9	12.5.....	11.1	1	19	95	6.645
9.....	11	11.1.....	9.1	1	20	100	—

*The reciprocal units are used in their reverse order (see text, footnote 5).

†Read from published probit tables (*19–21*).

type B (heterogeneous); see text

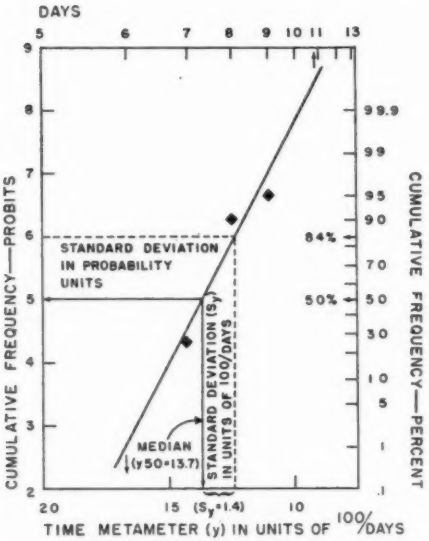
developing tumors at successive times and dilutions*

Dil. 10^{-4}		Dil. 10^{-5}		Dil. 10^{-6}		Dil. 10^{-7}		Dil. 10^{-8}	
Sample a b c d e	sum	Sample a b c d e	sum	Sample a b c d e	sum	Sample a b c d e	sum	Sample a b c d e	sum
3 3 1 3	10					†	†		
5 1 6 3 7	22	1 3 4 2 2	12	1	1				
1 5 1 3 2	12	3 3 2 5 4	17	3 3 2	8	1 1	2		
1 1	2	2 1 2	5	1 1	2	1	1		
1	1	3 2 1 1	7	1 1 2 4	8			1	1
		1	1	2 2 6 3 1	14	1 1 1 1	4		
1	1	1	1	1	1	1 2	3	1	1
		1 2	3	1 1 1 1	4	1	1		
				†1	†1	1	1		
				1	1				
	48		46		40		12		2
	50		50		50		50		50
	—		—		1		1		—
	50		50		49		49		50

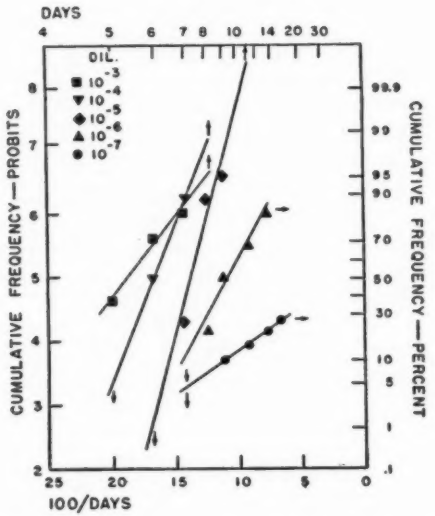
employed, and that methods of statistical analysis appropriate to the normal distribution are suitable for reliable analyses of the transformed responses. Estimation of the dose-group *mean* (which is identical with the median in a normal distribution) and of the *standard deviation* by the graphic procedures described by Bliss (*7*) are illustrated in text-figure 1.[6]

Fewer than 50 percent of the chickens developed tumors with dose 10^{-7} and the results for this group are therefore outside the range in which the latent period can be used as a reliable criterion of virus potency [see (*12*)]. They have been included in text-figure 2 in order to illustrate the flatter slope of the time-frequency curve usually observed with relatively low doses of the Rous sarcoma virus. A similar flattening of the slope at low-dose levels was also observed by Eckert, Beard, and Beard (*5*) with another chicken tumor virus (that causing myeloblastic leukosis). This phenomenon may therefore be largely responsible for the unreliability of latent-period data obtained with both of these viruses at relatively low dose levels.

[6] One of the reviewers of this paper pointed out that for samples of less than 30, such as the individual dose groups of table 1 which contain only 20 individuals, it would have been preferable to use rankits instead of probits in making the tests of normality, as well as for estimating the means and standard deviations. (See "Outline of Biometry," by C. I. Bliss and D. W. Calhoun, Yale Co-operative Corporation, 1954, for a discussion of the method of rankits.)



TEXT-FIGURE 1.—Graphic analysis of time-frequency data including estimation of the mean (median) and standard deviation in time metameter units (see text). The arrows pointing downward and upward represent 0 and 100 percent frequencies, respectively, for which probit equivalents are indeterminate.



TEXT-FIGURE 2.—Time-frequency curves for all dose groups within the significant range. Data of type A, table 1 (see text).

The results obtained with the two strongest doses (dilutions 10^{-1} and 10^{-2}) of table 1 are seen to be beyond the upper limit of the significant dose range, since most of the chickens had developed tumors by the time of the first examination on the 5th day. As pointed out in a preceding section, it is not practical to examine the chickens at intervals of less than 1 day, and, for all practical purposes, the latent-period response is limited at about the 5th day following virus inoculation. The data of the two highest dose groups (10^{-1} and 10^{-2}) have therefore not been included in text-figure 2, nor subjected to further analyses.

The means and standard deviations of all dose groups within the significant range are summarized in table 4.

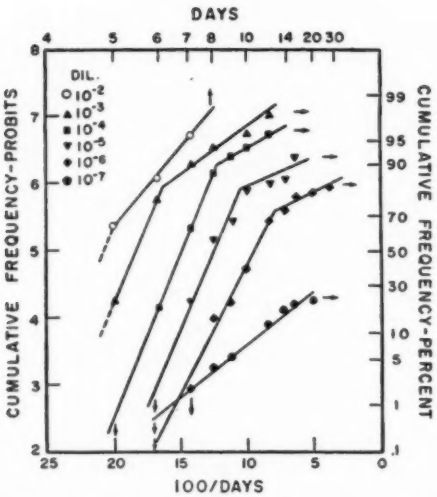
Heterogeneous chicken responses (data of type B, table 2).—The tumor responses of the respective dose groups are given in detail in table 2. Preliminary analyses of the latent-period results by graphic methods may be carried out in exactly the same way as described in the preceding section, the total number of chickens in a given group (*i.e.*, including those which failed to develop tumors) being used for calculating the cumulative percentages. As is evident in text-figure 3, the frequencies in probability units do not follow single straight lines as in the preceding instance (see text-fig. 2), but the time-frequency curves break and follow a flatter slope after periods of time which vary with the dose.

This type of result is similar to that reported by Eckert, Beard, and Beard (*5*) for latent-period responses to the myeloblastic leukosis virus. It represents one of the types of biological truncation discussed by both Bliss (*7*) and Ipsen (*8*). A second type of biological truncation (represented by the horizontal arrows of text-fig. 3) was the complete failure of some of the chickens to develop tumors, even with the strongest doses of virus. According to the procedures described by both Bliss (*7*) and Ipsen (*8*), aberrant individuals of the former type are regarded for the purpose of statistical analysis as if they had failed to respond altogether, and further analyses of the data are confined to the initial limb of the distribution curve (*i.e.*, up to the point of break in slope, or "truncation").

Computational procedures involving tabled constants of truncated normal distributions have been described by both Bliss and Ipsen for obtaining corrected estimates of the *mean* and *standard deviation*, as well as the "relative weights" of the corrected estimates. The procedures are

TABLE 4.—*Summary of dose-group means and standard deviations within groups for latent-period data of type A (table 1)—(results within significant dose range—see text)*

Log dilution	Number of chickens with tumors	Mean $\frac{100}{\text{days}}$		Standard deviation	
		Calculated from data	Estimated graphically	Calculated from data	Estimated graphically
−3	20	18. 76	18. 8	3. 12	3. 8
−4	20	16. 69	16. 7	1. 56	2. 0
−5	20	13. 68	13. 7	1. 19	1. 4
−6	17	11. 18	10. 9	1. 70	1. 7



TEXT-FIGURE 3.—Truncated time-frequency distributions of heterogeneous data of type B, table 2 (see text).

fairly involved, however, and some knowledge of the intricacies of statistical methodology is required for their use. On the other hand, graphic estimates of the *mean* and *standard deviation* may be derived as illustrated in text-figure 1 from the initial steep segments *only* of the truncated curves. Such graphic estimates are satisfactory for most purposes, provided sufficient numbers of test animals are used to provide regularity of the plotted points (*7*). As was emphasized by Bliss (*7*), "When the data are moderately uniform and there is biological truncation, it is frequently a more profitable expenditure of time to increase the number in each curve or the number of curves and to rely upon graphic analysis than to carry the calculations beyond this stage."

In table 5 are listed uncorrected and corrected estimates of the *mean* and *standard deviation* at successive dose levels within the significant range. The former were based upon all positive chickens, including those with aberrantly long latent periods but omitting completely refractory individuals. [This was the procedure employed in a previous analysis of these same data (*14*).] The corrected values were derived by two methods: (1) the graphic method of Bliss (see above), and (2) the computational procedure of Ipsen (*8*), involving separate estimates of the standard deviation for different groups.

Dose-Response Relationships Based upon All Dose Groups

The dose-group *means* and *standard deviations* derived by appropriate procedures for the respective types of latent-period data (A or B), are summarized in tables 4 and 5. Their relationships to log dose[7] are

[7] See references (*15, 22-28*) for discussions of the advantages of the use of log dose as the "dose metameter" for analysis of dose-response data.

TABLE 5.—*Summary of significant numbers of chickens, dose-group means, and standard deviations within dose groups for latent-period data of type B (table 2)—(results within significant dose range—see text)*

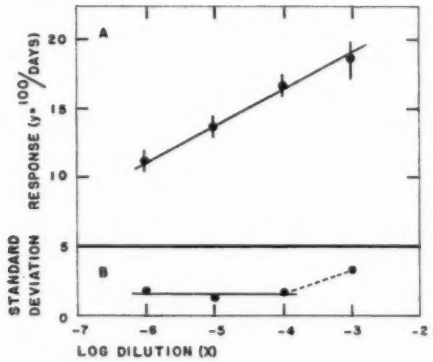
Log dilution	Uncorrected estimates			Corrected for truncation					
				Graphic (7)			Computed (8)		
	Number of chickens*	Mean $\frac{100}{\text{days}}$	Standard deviation	Number of chickens†	Mean $\frac{100}{\text{days}}$	Standard deviation	Relative weight of mean‡	Mean $\frac{100}{\text{days}}$	Standard deviation
−3	49	18. 32	3. 06	39	18. 3	2. 2	44. 4	18. 51	2. 56
−4	48	15. 14	2. 25	44	14. 9	2. 0	48. 0	15. 08	2. 12
−5	46	12. 70	2. 51	41	12. 4	2. 3	47. 7	12. 59	2. 28
−6	40	10. 34	1. 87	33	9. 5	2. 6	39. 7	8. 90	2. 71

*Total number with tumors.

†Number in frequency distribution up to point of truncation [see (7)].

‡Total number (n) in dose group multiplied by Ipsen's (8) appropriate tabled value, $w(\mu)$, as a weighting factor.

illustrated graphically in text-figures 4 and 5. It is seen that the response metameter, $y=\frac{100}{\text{days}}$, which proved suitable for statistical analyses of the individual latent-period responses within dose groups, also provides a linear relationship between dose-group mean and log dose.[8] The oblique lines of the upper charts of both figures represent computed regression lines based upon calculated estimates of the means. In the case of the heterogeneous data (text-fig. 5) the calculated estimates were corrected values derived by Ipsen's (*8*) method. As is evident from the latter figure, the uncorrected means (closed circles) deviate systematically from the remaining plotted points and from the corrected regression line. The corrected results obtained by graphic analysis did not differ significantly from those derived by algebraic procedures. The computed curve and its limits of error ($P=0.05$) are further illustrated in text-figure 6.



TEXT-FIGURE 4.—*A*. Relation of mean latent-period response in units of $\frac{100}{\text{days}}$ to the logarithm of dose. The vertical lines represent limits of error ($P=0.05$) for the plotted means. *B*. Relation of standard deviation to log dose. Data of table 1.

The Gaddum precision index, λ, was found from the homogeneous latent-period data, A, to be

$$\lambda=\frac{\bar{s}}{b}=\frac{2.03}{2.66}=0.76$$

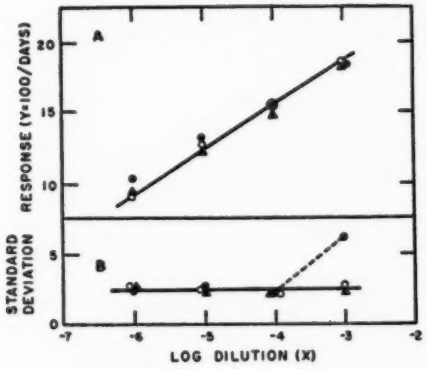
and from the heterogeneous data, B, to be

$$\lambda=\frac{\bar{s}}{b}=\frac{2.43}{3.12}=0.78$$

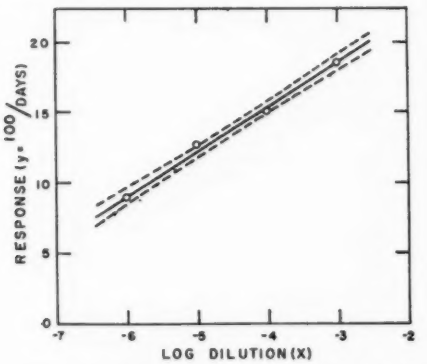
where $\bar{s}$ represents the average of the standard deviations (computed from their squares), and b represents the slope (or regression coefficient) of the log dose-response curve.[9] The use of this statistic for comparisons

[8] For discussions of the advantages of linear dose-response relationships for the purpose of statistical analysis, see references cited in footnote 7.

[9] See (*19*, *23*) for graphic method of estimating slope of the dose-response curve.



TEXT-FIGURE 5.—*A.* Relationship of mean latent-period responses, derived by different procedures, to the logarithm of dose.
●=uncorrected estimates.
○=corrected by the method of Ipsen (*8*).
▲=corrected by the graphic procedure of Bliss (*7*).
B. Standard deviation within dose groups estimated by different procedures. The symbols have the same significance as in chart A. They are plotted to avoid superposition at a given dose (see text).



TEXT-FIGURE 6.—Dose-response curve and limits of error (P=0.05) determined from the heterogeneous latent-period responses of tables 2 and 5 after correcting for the truncation (see text).

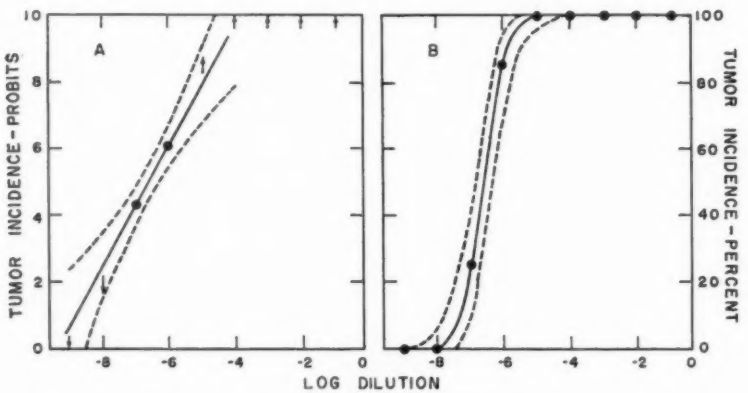
of relative precision, and for making "orienting estimates" of the numbers of test animals required for bioassays of specified accuracy, has been previously discussed (*15*).

Tumor-Incidence Response

Homogeneous Quantal-Response Data of Type A (Tables 1 and 6)

The percentage responses of successive dose groups of table 1 are summarized with respect to tumor incidence in table 6. Since only two of the dilution groups (10^{-6} and 10^{-7}) yielded percentage values between

0 and 100 percent, the choice of a suitable mathematical model for analysis of the results could not be made from the data of this experiment alone. However, previous studies [see table 1 and text-figs. 1 and 4 of reference (*16*)] involving larger numbers of chickens in the lower dose groups, as well as additional data on hand, indicate beyond question that the normal curve is satisfactory for the analysis of homogeneous tumor-incidence data of the present type obtained with the Rous sarcoma virus. The results of table 6 are plotted in terms of probability units (probits), and in percent, in the left and right charts, respectively, of text-figure 7. The arrows pointing downward and upward in the left chart represent 0 and 100 percent responses for which empirical probit equivalents are indeterminate. It is seen that the regression of probits on log dose is adequately represented by a straight line and that, for the purposes of further statistical analysis of the data, the quantal responses of individual chickens (in terms of the log dose equivalents) may be considered as belonging to a homogeneous normal distribution [*cf.* (*19, 20, 22–28*)]. Linear dose-response curves of this type may be further used for parallel-line assays [*e.g.*, under circumstances in which the dose-response curves are relatively flat, or in which the strongest materials yield less than a 50 percent response—*cf.* (*16*)] as well as for other purposes (see preceding section); or, the probit transformation may be used as a convenient method of estimating the mean (ED50) and the standard deviation (λ) in log dose units as illustrated in text-figure 8. The latter statistics are those most generally employed for comparing potencies of different materials where the conditions are such that the entire dose range within which response changes from 100 percent to 0 percent can be bracketed with test-animal groups in the assay experiments.

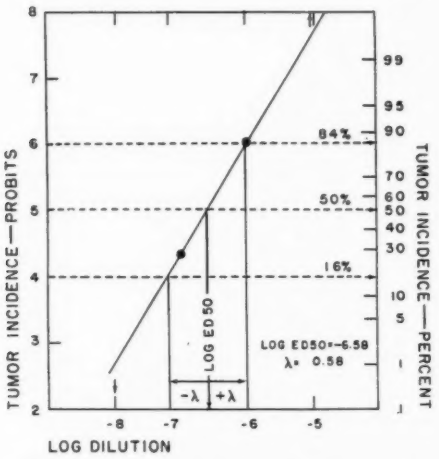


TEXT-FIGURE 7.—Computed dose-response curves for the homogeneous tumor-incidence data of tables 1 and 6.

A. Tumor incidence in probits.

B. Tumor incidence in percent.

The broken curves represent limits of error of the computed curves corresponding to $P=0.05$.



TEXT-FIGURE 8.—The estimation of log ED50 and λ by graphic methods (see text).

TABLE 6.—*Summary of homogeneous tumor-incidence data of table 1*

Log dilution (x)	Total number* in group (n)	Number with tumors (*positive*)	Percent positive	Empirical probit†
−1	18	18	100	—
−2	20	20	100	—
−3	20	20	100	—
−4	20	20	100	—
−5	20	20	100	—
−6	20	17	85	6.04
−7	20	5	25	4.32
−8	19	0	0	—
−9	19	0	0	—

*Corrected for deaths without tumor during observation period (see table 1).
†Read from published probit tables (*19–21*).

The standard deviation in log dose units (λ) represents the precision index described by Gaddum (*26*) for quantal-response data of this type. The graphic estimate of λ is seen to be 0.58, which is practically identical with the value computed as the reciprocal of the maximum likelihood slope (b) of the regression line, to be

$$\lambda=\frac{1}{b}=\frac{1}{1.81}=0.55.$$

Since probit values derived from quantal responses do not have the same "relative weight" at all percentage levels, but an average weight of only about 0.5 [as compared with unity for most graded responses—*cf.* (*15, 19, 25, 28*)] within the significant response range, the number of test animals required for bioassays of a given accuracy will, for the quantal response, be about twice that required for most graded responses having the same precision index (λ). In terms of numbers of animals required for the

same accuracy, therefore, a precision index of $\lambda=0.55$ would be equivalent to $\lambda=0.55\sqrt{2}$, or $\lambda=0.78$, for graded responses of unit "weight"—*cf.* (*15*).

The use of the probit transformation in bioassays, or as a "research tool," has been described in detail by a number of authors (see references cited above). Other methods of analysis of quantal-response data, including that of Reed and Muench (*29*), have been discussed by Finney (*24*), Armitage and Allen (*30*), Cornfield and Mantel (*31*), and Cornfield (*32*).

Heterogeneous Quantal Responses of Type B (Tables 2 and 7)

The tumor responses of table 2 are summarized with respect to incidence in table 7. It is seen that the dose range within which the tumor incidence dropped from 100 percent to 0 percent extended over 7 $\log_{10}$ intervals. This wide spread of the tumor-incidence response indicates either that the dose-response curve had an exceptionally flat slope for this particular group of chickens, or that the test animals were heterogeneous, and did not all belong to the same population of variates. Were it not for the fact that the same standard virus preparation was used which yielded a steeper slope, and homogeneous variations, in another test (see data of type A), the possibility would have to be considered that the nature of the virus material (*e.g.*, presence of aggregates which dissociated on dilution) or the presence of interfering substances (*e.g.*, an inhibitor, or another contaminating virus) contributed to unusual spread of the results.

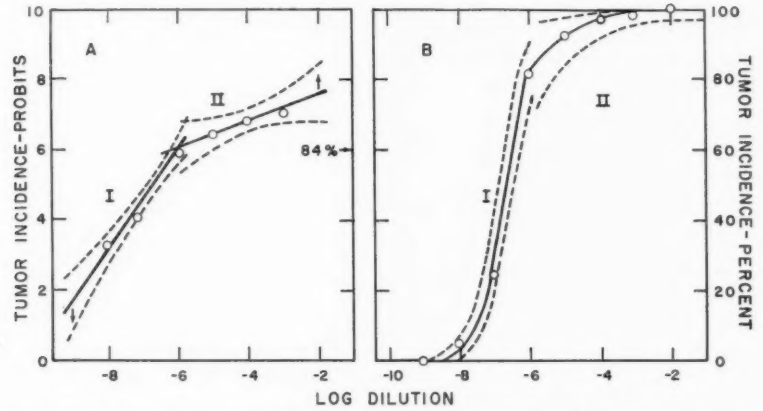
TABLE 7.—*Summary of heterogeneous tumor-incidence data of table 2*

Log dilution	Total number* in group	Number with tumors (*positive*)	Percent positive	Empirical probit†
−2	50	50	100	—
−3	50	49	98. 0	7. 05
−4	50	48	96. 0	6. 75
−5	50	46	92. 0	6. 41
−6	49	40	81. 6	5. 90
−7	49	12	24. 5	4. 31
−8	50	2	4. 0	3. 25
−9	49	0	0	—

*Corrected for deaths without tumor during observation period (see table 2).
†Read from published probit tables (*19–21*).

The nature of the dose-response relationship may be determined graphically as illustrated by the left chart of text-figure 9. The tumor incidence in probability units (probits) does not follow a single linear trend, as would be expected for a homogeneous normal population of variates, but the slope of the dose-response curve breaks at log dose −6.0 (the log of the dilution of virus) and follows a flatter slope at higher dose (lower dilution) levels. Since each plotted point of the figure is based upon 50 inoculated chickens, the deviation of the regression from a single straight line is highly significant. A curvilinear regression line could, of course, be fitted to the points, but the range of rapid change in slope would

be relatively narrow, and it is simpler for purposes of bioassay, to divide the total curve into 2 linear segments as indicated in the figure.



TEXT-FIGURE 9.—Computed dose-response curves for the heterogeneous tumor-incidence data of tables 2 and 7.

A. Tumor incidence in probits.

B. Tumor incidence in percent.

The broken curves represent limits of error of the computed curves corresponding to $P=0.05$.

It is apparent that the slope ($b=1.44\pm0.17$) of the initial segment (I) of the curve, although slightly flatter, does not differ significantly from that ($b=1.81\pm0.34$) observed for the homogeneous responses (see text-fig. 7), and that the precision index computed as the reciprocal of the slope for segment I, *i.e.*,

$$\lambda=\frac{1}{1.44}=0.69,$$

does not differ significantly from the value ($\lambda=0.55$) found for the homogeneous responses.

The second segment (II), on the other hand, has a slope of only $b=0.40$, and a standard deviation (in log dose units) of $\lambda=2.50$. The correlation of response and dose within this segment of the curve is therefore so slight as to make the results of little use for accurate bioassay purposes [*cf.* (*15*)].

Extrapolation of the initial limb (I) of the curve as a straight line would lead to the expectation of 100 percent responses at all dose levels above about log -5.0. Since chickens with aberrantly long latent periods were also observed (text-fig. 3) at these same higher dose levels, it is probable that the same factors which were responsible for the latter phenomenon also accounted for the "unexpected" completely refractory chickens which may simply have represented extremes among the aberrantly resistant individuals. This association between truncated time-frequency responses and truncated tumor-incidence curves has been

confirmed in two additional sets of similar data in which adequate numbers of chickens (50 or more per dose group) were available for critical analyses.

The division of the total dose-response curve into two segments for the purpose of analysis follows a precedent introduced by Bliss (*33*) for dealing with a change in slope caused by factors of another sort.

Discussion

The methods of graphic analysis exemplified herein not only serve to illustrate the underlying principles by which reliable statistical analyses can be carried out on data of the present types, but they also represent actual working procedures for obtaining approximate estimates of dose-group means, standard deviations, time-frequency curves, and dose-response curves, which may be used without further refinement for many biological research purposes. The analytical principles represented are those which were developed in the fields of pharmacology and biological standardization for the bioassay of drugs, hormones, vitamins, and other biologically active chemical compounds. The exacting demands of biological standardization are not always feasible or necessary, however, when the same principles and methods are used as research tools for biological studies on viruses.

First, for example, a single particle of virus can, under certain conditions, initiate a viral lesion which leads to a biological response (*i.e.*, after "amplification" of the initial reaction caused by the small stimulating dose through the process of viral multiplication). Even if there should be no variation between biological test units, therefore, their quantal responses would have an inherent variability conditioned by the chance distribution of virus particles in the unit inoculums. As was previously pointed out (*15*), the lower limiting value of the Gaddum precision index, λ [see above, and (*15*)], would be about 0.5, on an average, as a result of the error of sampling of virus particles alone. This happens to be the upper limit of the tolerated range of λ for purposes of "accurate" biological standardization, higher values being regarded as indicative of "inaccurate assays" (*25*). Thus, the accuracy of virus assays in animals begins with the upper tolerated limit for biological standardization of drugs, insofar as the magnitude of experimental error is concerned. As was also demonstrated previously (*15*), the value of λ for neoplastic virus-host systems may range as high as 1.2, or even higher in exceptional instances. This means that the experimental errors associated with virus bioassays in living animals are of a higher order of magnitude than those associated with the assay of drugs and other chemical compounds. Even in instances in which it is possible to assay virus activity by more accurate methods, *e.g.*, by "plating" methods involving counts of foci of unit particle activity [*cf.* (*1*)], studies on the kinetics of host-virus interaction in intact animals would still require experimentation with systems having the lower precisions (higher λ values) indicated above. The same standards of accuracy as are considered imperative for biological standardization are therefore not obtainable with practical numbers of test animals in most investigations on animal viruses involving intact hosts.

Second, because of the "amplification" of the reactions to extremely small doses of virus, the same qualitative end result (*e.g.*, development of a tumor) may be observed in response to various doses extending over an extremely wide range, *e.g.*, 7 or more $\log_{10}$ intervals in the instance of the present agent. Also, quantitative criteria of virus potency (*e.g.*, latent period; incidence of positive reactions) are usually highly correlated with dose over at least 2 and frequently 3 or more $\log_{10}$ intervals. As a result, larger increments of change in relative potency have quantitative significance, and larger experimental errors can be tolerated in quantitative biological work with viruses than with other, nonreproducing, types of agents. The situation is therefore analogous to the consideration of changes in hydrogen-ion concentration on a logarithmic scale (*i.e.*, in terms of *p*H). As in the latter instance, errors of several tenths of a $\log_{10}$ interval would appear absurd in terms of percent or in arithmetic values, but the results subject to such errors have a very real and useful quantitative meaning for these particular systems.

As has already been pointed out, the graphic approximations illustrated herein are sufficiently accurate for many types of quantitative studies involving variations in virus potency on a logarithmic scale (*e.g.*, those involving host-virus interactions). On the other hand, there are some purposes [*cf.* (*15*)] for which the required accuracy of virus bioassays may approach the limits necessary for biological standardization of drugs and other chemical compounds (*e.g.*, "confidence limits" not greater than about 25 to 50 percent). In these instances, exceptionally large numbers of test animals may have to be used [*cf.* (*15*)], and determinations of the bioassay results would have to be derived by more accurate statistical procedures. The latter are described in detail in the references cited in connection with the various graphic illustrations.

Summary

Typical data are shown and principles of their analysis are illustrated for biological responses to the Rous sarcoma virus involving one subcutaneous inoculation site on each test chicken. The procedures illustrated include tumor responses of both the measurable (latent-period) and quantal (tumor-incidence) types, (*a*) when the chicken susceptibilities are homogeneous, and (*b*) when they are heterogeneous.

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The Brain of Newly Hatched Chicks as A Host-Virus System for Biological Studies on the Rous Sarcoma Virus (RSV) [1,2]

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Vazquez-Lopez (*1*) studied the growth and histology of Rous sarcoma (chicken tumor I) inoculated into the brain of chickens which were 3 to 5 months of age. He reported that when grafts were implanted into the brain the tumors grew in the form of nodules without invasion of the surrounding nerve tissue, which was compressed and displaced. However, when cell-free material was inoculated, the tumors which arose grew diffusely and invaded the brain. Chickens inoculated with cell-free material died later than those inoculated with tumor tissue. The microscopic appearance of the Rous sarcoma in chicken brain was similar to that presented by intramuscular or subcutaneous tumors. Tumors growing in chicken brain contained the two typical types of cells, namely, the elongated cell which is similar to the fibroblast and the round, smaller cell which resembles the macrophage. The cellular types were the same whether the transmission was made by graft or by inoculation of cell-free material.

Duran-Reynals (*2*) studied the reactions of 2- and 7-day-old chicks to intracerebral inoculations of Rous sarcoma filtrates as a complement to his studies on lesions of the central nervous system produced by a duck variant of the Rous sarcoma virus (RSV). The pathologic findings in the brain were much the same as those described by Vazquez-Lopez in older chickens. Duran-Reynals also showed that the virus could be propagated in the chick brain through at least five successive passages.

Although pathologic studies of the infected brain tissue were not made in the present investigation, Dr. W. H. Eyestone, of the National Cancer Institute, has kindly examined the brain of one chick killed when moribund 10 days after intracerebral inoculation with RSV. No gross lesions were observed in the brain. Histologic examination revealed an abundance of Rous sarcoma cells similar to those described by Vazquez-Lopez

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[3] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

and by Duran-Reynals beneath the meninges but not invading the brain parenchyma in the sections studied.

Newly hatched chicks have recently been found to be useful experimental animals in the study of rinderpest virus (*3*), rabies virus (*4*), diphtheria toxin (*5*), equine encephalitis virus (*6*), and foot-and-mouth-disease virus and *Coxiella burnetti* (*7*). The data presented below indicate (*a*) that Rous sarcoma virus propagates in the brain of newly hatched chicks and kills them in relation to dose, (*b*) that a prior inoculation of influenza B virus exerts a sparing effect on mortality resulting from RSV, and (*c*) that RSV in the brain of young chicks presents a new and useful host-virus system for biological studies on RSV.

Materials and Methods

General.—Rous sarcoma virus (RSV), also commonly referred to as the agent of chicken tumor I, was originally isolated by Rous in 1911 (*8*), and a substrain of it was obtained from the National Cancer Institute, where it has been maintained by serial passage in chickens. The LEE strain of influenza B virus was maintained by serial passage in the allantoic sac of embryonated eggs in the usual manner. Unsexed White Leghorn chicks were used throughout these studies. They were obtained from a reliable local hatchery known to be free from *Salmonella pullorum*.

Standard RSV.—The Rous sarcoma virus used in all experiments was from a common frozen lot, standard lot CT 581, the preparation and preservation of which have been previously described (*9*). It was a microsome fraction derived from isotonic citrate extracts of tumor tissue by differential centrifugation. Samples of the standard material were sealed under reduced pressure in small glass ampules and were preserved by freezing and storage in a CO_2 ice chest (−50 to −78° C.). The diluent for the standard RSV was saline containing 2 percent inactivated (56° C. for 30 minutes) normal horse serum.

Intracerebral inoculation of chicks.—Chicks were placed under light ether anesthesia and inoculated intracerebrally into the right hemisphere with 0.05 ml. of inoculum by means of a 1/8-inch 26-gauge needle. The point of inoculation was the center of an imaginary equilateral triangle, the base of which extended from the right eye to the base of the skull. The hypodermic needle was inserted to its hilt (1/8 inch). Each chick was removed from the anesthetic jar 2 seconds after it had collapsed to avoid overdosage with ether, which is a hazard because of the direct connection of the air sacs with the respiratory tree in the chicken. Mortality due to RSV was never observed before the 7th day after inoculation, even with inoculums of highest potency.

Subcutaneous inoculation into the wing web.—Two-tenths ml. amounts were injected subcutaneously into the wing web using a 3/4-inch 26-gauge hypodermic needle. The ventral surface of the wing was swabbed with isopropanol and the needle was inserted through the muscle into the subcutaneous tissue of the wing web, thus avoiding leakage. With the exception of an experiment in which the responses were compared in

opposite wings of the same chicken, only the left wing was inoculated. Multiple tumors 0.25 to 2.0 mm. in diameter appeared after 4 or more days, depending on the potency of the inoculum. Each bird was examined daily by swabbing the wing web with isopropanol and examining the wing web with both direct and transmitted illumination.

Serial passage of RSV in chick brain.—Intracerebral passage of RSV in chicks was initiated with standard RSV diluted 1:10 in saline containing 2 percent inactivated normal horse serum. Brain tissue was removed aseptically from chicks which were moribund or prostrate 8 or more days after inoculation of RSV. The brain tissue was immediately placed in a screw-capped vial and stored at −70° C. Frozen brain tissue was thawed, ground in a mortar with sand, and suspended in enough saline containing 2 percent horse serum to make a 10 percent suspension of brain tissue by weight. This suspension was clarified by centrifugation at 2,000 r.p.m. for 10 minutes, and 0.05 ml. amounts of the supernatant fluid were inoculated intracerebrally into 10 or more chicks. Care was taken to ensure that the period of time between removal of the frozen brain tissue from the dry-ice chest and inoculation of the last chick did not exceed 2 hours.

Although the centrifugation procedure employed in these earlier experiments does not assure the complete removal of all intact tumor cells which may possibly have escaped disintegration during freezing, thawing, and grinding, the relatively small numbers of such cells which may have been present would not be expected to alter appreciably the biological responses to highly potent virus suspensions such as those involved in the present investigation. This was confirmed in later brain-passage studies in which precautions were taken [*cf.* (*10*)] for the removal of possible intact cells.

Criterion of specificity of mortality in chicks inoculated intracerebrally with RSV.—Almost invariably chicks dying after intracerebral inoculation of RSV showed a hemorrhagic area from 0.5 mm. to 10 mm. in diameter in the meninges at the site of inoculation into the brain. Thus, all but 107 of 2,629 (4.1%) dead or moribund chicks inoculated with various dilutions of RSV showed this typical lesion. Conversely, only 5 of 278 chicks (1.8%) surviving inoculation with very dilute inoculums showed any gross evidence of hemorrhage in the meninges. Consequently, all chicks were examined at the time of death or sacrifice for the presence or absence of the typical hemorrhagic lesion in the meninges at the site of inoculation. Further studies of its nature are in progress.

Propagation of RSV in embryonated eggs.—Eggs in the 8th day of embryonic development were inoculated onto the chorio-allantoic membrane with standard RSV diluted 10^{-1} and incubated for 7 to 10 days at 36° C. Membranes with well-developed lesions were immediately harvested from living embryos and were stored in screw-capped vials at −70° C. Serial passages of RSV were made using a 10 percent suspension of the membranes from frozen stock. The membranes were ground in a mortar with sterile sand, suspended in saline, and clarified by centrifugation at 2,000 r.p.m. for 15 minutes. Care was taken to ensure that

no more than 2 hours elapsed between removal of the frozen membranes from the dry-ice chest and inoculation of the last egg.

Analysis of the data.—The reciprocal of time in days $\times 100 \left(i.e., y = \frac{100}{\text{days}} \right)$ was found to be a suitable transformation for analysis of both the tumor latent period and the time-to-death responses. The dose-group means in terms of this function were observed to be linearly related to the logarithm of dose, as had previously been reported for similar latent-period responses (*9, 11–13*).

The dose-response data were analyzed by the graphic methods recently illustrated in detail for similar responses to the Rous sarcoma virus (*10, 11*), including that of correcting for the heterogeneity observed in some experimental lots of chickens due to the occurrence of aberrant, highly resistant chickens. As has been suggested by Eckert, Beard, and Beard (*14*), the aberrant chicken responses were considered as belonging to the truncated segments of truncated normal distributions, and graphic procedures described by Bliss (*15*) for the analysis of such truncated distributions were applied.

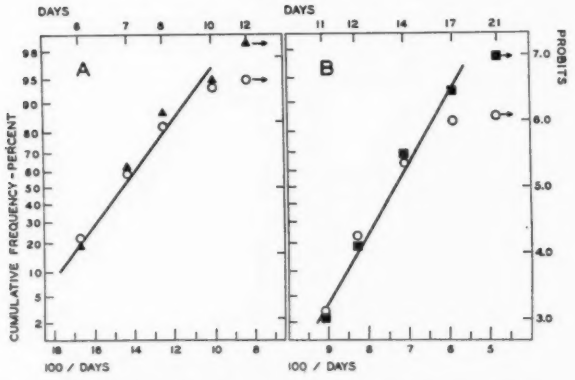
For comparing groups of chickens which had received the same dose of RSV but which had been subjected to different experimental treatments [*e.g.*, (*a*) control and (*b*) prior injection with a modifying agent], the frequencies of individuals responding at different times were accumulated for successive time intervals, converted to percentages, and plotted on probability paper.[4] The time-frequency relationships were linear in this form, thus permitting graphic estimation of the means (medians) and standard deviations of the distributions from the positions and slopes of the respective lines [*cf.* (*11, 16*)].

The comparisons of relative potency among different RSV preparations were made by plotting the dose-group mean (median) responses against logarithm of dose (dilution) and observing the horizontal distance between parallel dose-response curves [*cf.* (*10, 11, 17, 18*)].

Results

The data presented in text-figure 1 compare the responses of newly hatched chicks to RSV inoculated intracerebrally and into the wing web. A single dosage level of RSV (standard RSV diluted 10^{-2}) was used to inoculate 3 groups of birds as follows: group 1 (triangles) consisted of 57 birds inoculated with RSV into the wing web; group 2 (squares) consisted of 39 birds inoculated intracerebrally with RSV; and group 3 (circles) consisted of 99 birds, each of which was inoculated both intracerebrally and into the wing web with RSV. Section A of text-figure 1 shows the latent period for tumor response in the wing of groups 1 and 3, and section B shows the cumulative mortality of chicks inoculated intracerebrally (groups 2 and 3). Both the latent period and mortality are plotted against time in units of 100/days. The data show (*a*) that mortality following intracerebral inoculation of RSV occurred later than the appear-

[4] No. 32,451 (normal ruling); Codex Book Co., Norwood, Mass.



TEXT-FIGURE 1.—Comparison of chick responses to RSV inoculated intracerebrally and into the wing web. *Section A:* Latent period of tumor response in the wing. *Section B:* Time to death, in days, after intracerebral inoculation of RSV. ▲=RSV inoculated only into the wing web; ■=RSV inoculated intracerebrally; ○=RSV inoculated both intracerebrally and into the wing web (see text).

ance of tumors in birds inoculated into the wing web, and (*b*) that inoculation of RSV by one route did not alter the response of chicks to simultaneous inoculation by the other route when the same individuals were inoculated both intracerebrally and subcutaneously into the wing web.

Table 1 shows the relationship between the latent period of wing-tumor response and mortality following intracerebral inoculation of RSV in the same individuals. These data were collected from the records on group 3 in text-figure 1. It is clear that when the same birds received simultaneous inoculations of the same dose of RSV both intracerebrally and into the wing web, the time of appearance of the tumor in the wing was not a

TABLE 1.—*Relationship between responses of chicks to RSV inoculated simultaneously into the brain and into the wing web in the same individuals**

Mortality on day:	Tumor response—latent-period frequencies (*days*)							Total
	6	7	8	9	10	11	Neg.	
11	3†	7	—	—	—			10
12	7	10	2	—	1			20
13	7	13	6	1	1			28
14	3	5	5	—	1			14
15	1	1	5	—	—			7
16	—	—	2	—	4			6
17	—	—	—	—	1			1
18	—	—	—	—	—	1		1
Living without tumor on 21st day	1	2	2	0	1	0	6	12
Total	22	38	22	1	9	1	6	99

*Inoculum: standard RSV diluted 10^{-3}.

†Three chicks developed tumors in the wing on the 6th day after subcutaneous inoculation of RSV and died 11 days after intracerebral inoculation of RSV.

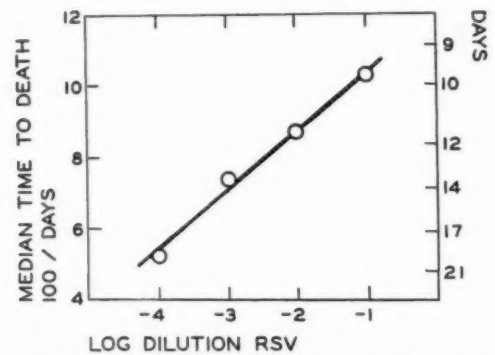
reliable indication of when *that particular individual* would succumb to the intracerebral inoculation, although there was a highly significant correlation between the averages of the two types of responses ($r = 0.88$; $P < 0.01$). It would appear that the factors which influence resistance and susceptibility of chicks to these two types of response, while related on an average basis, are not identical in their variability with respect to individual chickens. On the other hand, the data shown in table 2 indicate that the latent periods for wing-tumor responses in chicks inoculated simultaneously into both wing webs with the same dose of RSV were much more regular and were more highly correlated ($r = 0.96$; $P < 0.02$). These data in newly hatched chicks are in agreement with those obtained with birds 2 to 4 weeks of age (*12*).

TABLE 2.—*Relationship between responses of chicks to RSV inoculated simultaneously into the left and right wing webs of the same individuals**

Tumor response—latent-period frequencies						
Left wing (*days*)	Right wing (*days*)				Neg.	Total
	5	6	7	8		
5	6†	5	—	—	2	13
6	—	19	2	—	1	22
7	—	—	5	3	1	9
8	—	—	2	2	1	5
Neg.	—	—	—	—	1	1
Total	6	24	9	5	6	50

*Inoculum: standard RSV diluted 10^{-2}.
†Five chicks developed tumors in both wings 5 days after inoculation of RSV.

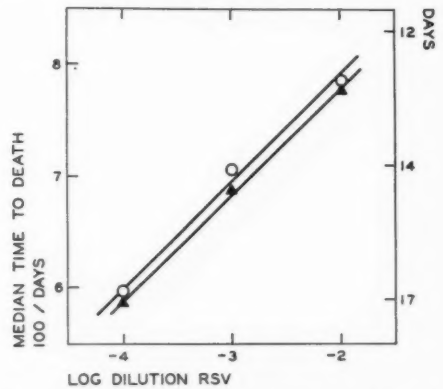
Text-figure 2 shows the response of chicks to intracerebral inoculation of successive tenfold dilutions of RSV from the fifth egg passage. In this experiment chorio-allantoic membranes with well-developed lesions were removed from frozen stock and a 10 percent suspension by weight was



TEXT-FIGURE 2.—The linear relationship of mean (median) time to death, in units of 100/days, to the logarithm of dose.

prepared. Three additional decimal dilutions of the tissues were made, and 0.05 ml. amounts of each dilution were inoculated intracerebrally into groups of 25 chicks each. The birds were observed for a period of 4 weeks, and the time required to kill 50 percent of the birds was estimated graphically for each dilution (see Materials and Methods). The median time to death, in units of 100/days, is plotted against the log of the dilution of RSV. It is clear that the relationship between them was linear in this experiment. Further studies are in progress to determine whether time to death following intracerebral inoculation into newly hatched chicks constitutes an efficient bioassay method for the comparison of potencies of different preparations of RSV.

Text-figure 3 shows the responses of chicks to intracerebral inoculations of RSV from the fifth serial passage in chick brain and to an aliquot of the same lot of frozen standard RSV as that which was originally used to



TEXT-FIGURE 3.—Response of chicks to the intracerebral inoculations of successive dilutions of RSV from the fifth passage of chick brain and of standard RSV. ○= Fifth brain passage RSV; ▲=standard RSV (see text).

initiate the intracerebral passages. Both standard RSV and brain tissue from the fifth intracerebral passage were diluted 10^{-2}, 10^{-3}, and 10^{-4}, respectively, and 0.05 ml. amounts of each of these dilutions were inoculated intracerebrally into groups of 35 chicks each. This experiment was identical in design to that just described, and time to death was plotted as described above against the log of the dilution of the two preparations of RSV. It is clear that after 5 passages in chick brain the potency of RSV was identical with that of the standard RSV used to initiate the intracerebral passages. An additional 9 passages, making a total of 14 serial passages of RSV in chick brain, have been completed to date with no reduction in potency. This indicates that RSV multiplies readily in chick brain.

The data presented in table 3 show that RSV from the fifth serial passage in chick brain was capable of eliciting a tumor response in the wing web which was comparable with the same lot of standard RSV used to

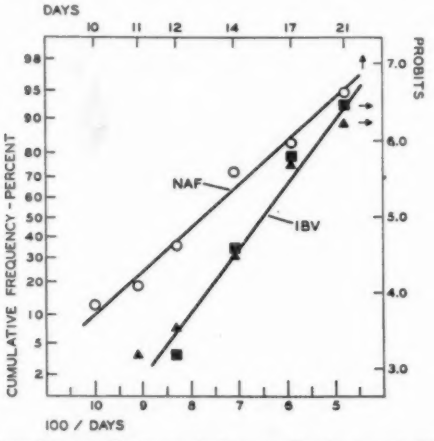
initiate the intracerebral passages. In this experiment standard RSV and frozen brain tissue were diluted 10^{-2}, 10^{-3}, and 10^{-4}, respectively, and 0.2 ml. amounts of each dilution were inoculated into the wing web of groups of 35 chicks each. Each bird was examined daily for the appearance of tumors and the LP50 (time in days to produce tumors in 50 percent of the chicks) was estimated graphically. The data clearly indicate that five serial passages in chick brain did not alter the capacity of RSV to produce tumors in the wing web.

Text-figure 4 presents data from a typical experiment showing that a prior intracerebral inoculation of influenza B virus exerted a sparing effect on mortality of chicks inoculated intracerebrally with RSV. Three groups of 54 chicks each were inoculated intracerebrally with 0.05 ml. amounts of normal allantoic fluid, influenza B virus, and influenza B virus heated to 56° C. for 60 minutes, respectively. Twenty-four hours later, each of these chicks was inoculated into the opposite hemisphere with 0.05 ml. of standard RSV diluted 1:10. In addition, 2 groups of 54 chicks each received a single similar intracerebral inoculation of influenza B virus and heated influenza B virus, respectively. Mortality in these latter 2 groups was negligible (2.5%). This experiment was terminated 27

TABLE 3.—*Tumor response in chicks inoculated into the wing web with RSV from the fifth serial passage in chick brain*

RSV	RSV diluted		
	10^{-2}	10^{-3}	10^{-4}
		*LP50**	
Standard RSV	4.2	4.9	5.6
Fifth brain passage	4.2	5.3	5.8

*LP50=median latent period or time in days to produce tumors in 50 percent of chicks inoculated.



TEXT-FIGURE 4.—Interference between influenza B virus and RSV in chick brain. ○=Prior injection of normal allantoic fluid; ■=prior injection of viable influenza B virus; ▲=prior injection of heated influenza B virus. $P<0.01$ (see text).

days after infection, and the results are presented graphically in text-figure 4. It is evident that both viable and heat-inactivated influenza B virus were equally capable of exerting a definite but transient sparing effect on mortality resulting from the intracerebral inoculation of RSV. The probability that this delay in mortality was the result of chance variation was less than 1 percent, as determined from the graphic estimates of the standard deviations (see Materials and Methods). It would appear that the sparing effect of influenza B virus is best explained as viral interference rather than as an oncolytic action of influenza B virus on the tumor cells, since heat-inactivated influenza B virus, known to be a potent interfering agent (*18, 19*), was as effective as viable influenza B virus. An experiment, similar in design to the one just described, failed to demonstrate any sparing effect when 0.1 ml. of 15-fold concentrated influenza B virus was injected into the wing web 24 hours before inoculation of 0.1 ml. of standard RSV diluted 1:100 (*20*). It would appear that interference between influenza B virus and RSV could only be demonstrated in the brain. All attempts to enhance the interference phenomenon shown in text-figure 4 were unsuccessful. Thus, in similar experiments further dilution of RSV through 10^{-4}, 20-fold concentration of influenza B virus prior to heat inactivation, and inoculation of influenza B virus 3 days before or 1 day after RSV, all either failed to improve the situation or were much less effective.

Discussion

The biological aspects of RSV and the sources of experimental variation and their control have recently been reviewed (*21, 22*). The development of stable standard preparations of RSV (*9*) showed the importance of controlling variations in host response in planning experiments and made it possible to estimate the potency of unknown preparations by comparing them with a standard preparation of RSV. Thus, it was possible by this method to show that the amount of RSV recoverable from tumor tissue was a function of the initiating dose of virus (*10*).

The studies reported here describe another host-virus interaction which may prove to be of value in biological studies with RSV. It is of interest that influenza B virus, known to interfere with many other viruses (*19*), also interferes with RSV in chick brain but not in the wing web. Too little is known of the pathogenesis or of the characteristics of RSV serially propagated in chick brain to determine as yet the potential usefulness of this system. The linear relationship of time to death, in appropriate metameter units (*i.e.*, 100/days), with log dose of RSV obtained in several experiments with standard RSV, brain tissue, and chorio-allantoic membranes suggests that this system may also prove to be useful in bioassay and in studies concerned with host-virus physiology.

Summary

Rous sarcoma virus (RSV) was found to propagate serially in the brain tissue of newly hatched chicks and to kill them in relation to dose. RSV

derived from the fifth serial intracerebral passage was capable of inducing tumors when it was inoculated subcutaneously in the wing. The data suggest that the rate of death (*i.e.*, the reciprocal of the time to death) following intracerebral inoculation was linearly related to the log of the dose of RSV. When the same birds received simultaneous inoculations of the same dose of RSV intracerebrally and subcutaneously into the wing web, the time of appearance of a tumor in the wing was not a reliable indication of when the particular individual would succumb to the intracerebral inoculation, although the two reactions were highly correlated on an average. Prior intracerebral inoculation of viable or inactivated influenza B virus exerted a sparing effect on mortality in chicks resulting from the intracerebral inoculation of RSV. This sparing effect resembles interference between viruses rather than an oncolytic action.

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Biological Studies on the Rous Sarcoma Virus. V. Preparation of Improved Standard Lots of the Virus for Use in Quantitative Investigations [1]

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The preparation and preservation of pure, concentrated, highly active extracts of the Rous sarcoma agent, as well as other viruses, have engaged the interest of investigators for a number of years.

Of the various physical and chemical techniques that have been employed in the separation of viruses from the normal components of the host tissues, the following have been found to be of value in purification studies on the Rous sarcoma virus: 1) chemical reactions of the protein portion of the entity, *e.g.*, salting out, formation of salts with heavy metals, protein-protein complex formations, enzymatic hydrolysis, adsorption, and subsequent elution of the virus or contaminating protein material from a suitable substrate; and 2) the physical technique of differential or alternating high- and low-speed centrifugation.

Although final purification of the agent of the Rous sarcoma has not as yet been achieved, work in this laboratory has shown considerable progress toward increasing the potency-nitrogen ratios of the final virus suspensions. With the use of citrate salts as stabilizing media (*1, 2*) in combination with techniques to be described below, it is now possible to produce standard lots of the virus having a higher degree of biological activity on a unit-of-nitrogen basis than were previously obtainable (*3*). The problem of increasing the relative purities and decreasing the variations among different lots of standard virus to be used in quantitative biological studies has been discussed by Bryan (*4*). The purpose of this report is to describe a physicochemical technique for the preparation of improved standard lots of virus for use in quantitative investigations. The method described herein employs three of the above-mentioned techniques, namely, preliminary hydrolysis of the intercellular cement by the enzyme hyaluronidase, formation of a protein-protein complex using protamine sulfate followed by tryptic digestion, and separation of the active virus from the nonactive material by differential centrifugation.

The use of protamine, a protein precipitant, has been found useful in purification studies on both plant and animal viruses. In this respect,

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[2] With the assistance of John P. Kvedar.

[3] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

Warren *et al.* (*5*) found that viruses fall into two groups: A) those which are precipitated by protamine, and B) those which after treatment with protamine remain in the supernatant. Employing the latter phenomenon, followed by tryptic digestion, Weil *et al.* (*6*) were able to separate from brain tissue a highly pure suspension of the encephalomyocarditis virus. Likewise, Briefs *et al.* (*7*), using the same procedure, separated from normal tissue components suspensions of two Group A Coxsackie viruses of high infective titer.

The viruses of tobacco mosaic (*8*), influenza A (*9*), and the Rous sarcoma are precipitated by protamine and are representative of the first group of viruses described by Warren *et al.* This reaction is considered in the purification procedure described herein.

Attempts to increase the relative purity of Rous sarcoma extracts by enzymatic hydrolysis of contaminating substances were carried out as early as 1927 by Baker and McIntosh (*10*). These investigators showed that the infectivity of filtrates was considerably enhanced if treated with a glycerine extract of pancreas tissue. This "stimulating" effect was evident at a *p*H of 6.0, a level other than optimum for tryptic activity. Although Claude (*11*) found that crude extracts of the Rous sarcoma were inactivated "rapidly and completely" by the proteolytic activity of trypsin, Amies and Carr (*12*), using a pancreas extract, "pancreatin absolute," were able after further centrifugation to obtain an active suspension suitable for serologic studies. Recent work by Carr and Harris (*13*) has shown that highly active "concentrates" of the Rous agent can be obtained by differential centrifugation of tumor extracts after treatment with crystalline trypsin. These workers also used hyaluronidase for reducing the high viscosity of the tumor extract.

The value of a mucolytic enzyme (hyaluronidase), as first recommended by Claude (*14*), and trypsin, as reported by Carr and Harris, has been repeatedly confirmed in this laboratory and is used in the purification procedure described in this report.

The principle of differential centrifugation has been widely used in virus-purification studies. References to investigations involving its use in the fractionation of Rous sarcoma extracts have been given in reviews by Harris (*15*) and Bryan (*4*).

Details of the purification procedure and some results of biological and biochemical studies on "partially purified" standard lots of the Rous sarcoma virus are presented herewith.

Materials and Methods

Solutions and buffers.—Approximately isotonic (0.15 *M*) potassium-citrate solution was made up from the salt ($K_3C_6H_5O_7 \cdot H_2O$) as required.

All buffers were dilutions of 1.0 *M* stock solutions. Appropriate *p*H was obtained by the combination of equimolar solutions of citric acid ($H_3C_6H_5O_7 \cdot H_2O$) and sodium citrate ($Na_3C_6H_5O_7 \cdot 2H_2O$). Triple distilled water, from an all-glass apparatus (*16*), was used as the solvent.

Freezing of virus suspensions.—Using the method described by Bryan

et al. (*3*), 0.15 ml. aliquots of the virus suspension, in 60 × 6 mm. ampoules, were sealed while under reduced pressure and the samples frozen in a block of dry ice. Until used, the ampoules were stored in a frozen CO_2 box (−50 to −76° C.).

Thawing and preparation of virus samples for bioassay.—As previously reported (*3*), the procedure found to be most efficient in the handling of the standard samples is as follows: 1) Ampoules removed from the frozen CO_2 box are immersed first in 95 percent ethyl alcohol and then in ether, 2) wiped dry with a sterile gauze sponge, 3) opened with glass-tubing cutters and, while the material is still in the frozen state, each ampoule is placed in a specimen vial containing 14.85 ml. of cold buffer solution. A capillary-tipped pipette is used to disperse the virus as it reaches the temperature of the diluent.

In the experiments described in this report the diluent used was 0.05 *M* sodium-citrate buffer at a *p*H of 6.7, containing hyaluronidase in the amount of 1 mg. per 100 ml.

Biological tests.—Appropriately diluted virus material in 0.2 ml. amounts was inoculated into the left wing-web area of 3-week-old New Hampshire Red chickens. The test animals had been maintained in this laboratory from 1 day of age on a standard diet of Purina Checker-etts and water.

A previously determined schedule for examination of the inoculated sites was carried out. The reciprocal of the latent period in days (× 100) was the criterion for biological response (*17*, *18*).

Chemical analyses.—As required, several ampoules containing 0.15 ml. of the frozen standard material were thawed at room temperature, pooled, and sampled for the analyses as described below.

Nitrogen: Total nitrogen and protein nitrogen were determined by a micromodification of direct nesslerization (*19*).

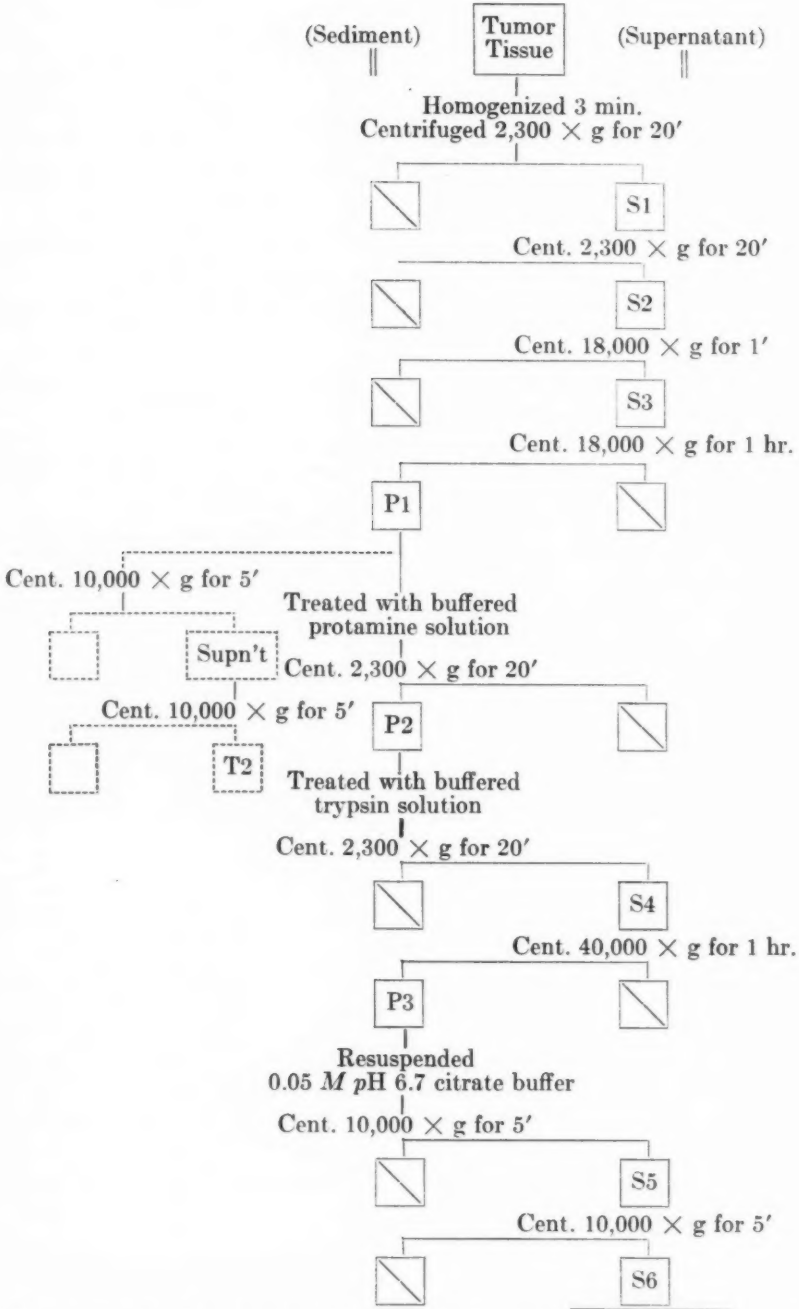
Phosphorus: Phosphorus determinations were made by a modification of the method described by Fiske and Subbarow (*20*).

Nucleic acid: The nucleic acids were extracted from the sample with hot perchloric acid (*21*). The acid precipitate was resuspended and sampled for protein nitrogen, which was determined as described above. Pentose nucleic acid (PNA) was determined by the Mejbaum orcinol reaction (*22*). Desoxypentose nucleic acid (DNA) was determined by the diphenylamine reaction of Dische (*23*). Absorption of the nitrogenous bases, in the acid extract, was measured in terms of optical density at 2,600 Å in a model DU Beckman spectrophotometer. These values, when referred to a standard absorption curve of either thymus or yeast nucleic acid, serve to validate the analytical methods for the nucleic acids enumerated.

Procedures and Results

Preparation of Standard Lots of Virus

The essential steps and operative procedures are presented diagrammatically in the flow chart shown in text-figure 1, the first five steps of which describe a preliminary separation of the suspended active fraction



TEXT-FIGURE 1.—Flow chart for preparation of a partially purified Rous-sarcoma-virus suspension (see text).

from tissue debris. This preliminary separation has been previously described as material T2 in a thesis (*24*) and briefly summarized in a recently published report (*3*) in connection with the preparation of partially purified standard virus lots CT 559 and CT 581.[4]

Thirty-five grams of bacteria-free tumor tissue which had been stored in a frozen CO_2 box (approximately −50 to −76° C.) was thawed at room temperature. A 6.6 percent suspension of tissue in 0.15 *M* potassium citrate containing 1 mg. of hyaluronidase [5] per 100 ml. was allowed to digest for 1 hour at room temperature with occasional mixing in the Waring Blendor (Model FC114). At the end of the digestion period the material was homogenized for 3 minutes. The homogenate was then cleared of nuclei and large cell fragments by centrifugation at 2,300 × g for 20 minutes [6] in an International refrigerated centrifuge, using rotor No. 845. The supernatant (S1) [7] was carefully pipetted off, care being exercised not to disturb the sediment or to include the thin top layer of lipide material. Fraction S1 was then recentrifuged, as above, to insure a more complete separation of larger cell fragments from the more particulate elements of the cytoplasm. The supernatant (S2) from this second centrifugation was pipetted off leaving a sediment and upper layer as residual material. Fraction S2 was cleared of mitochondrial fragments and larger cellular particulates by centrifugation at 18,000 × g for 1 minute in a Servall angle centrifuge.[8] After centrifugation the pellet material was considered sufficiently well packed to warrant pouring off the supernatant. This supernatant (S3) was then centrifuged in a Servall centrifuge at 18,000 × g for 1 hour to sediment the crude microsome fraction.[9]

This fraction (P1) was resuspended in 100 ml. (one fourth the centrifuged volume) of 0.005 *M* sodium-citrate buffer at a *p*H of 7.5. To this was added protamine sulfate in an amount equivalent to 3 mg. per ml. A Servall Omni-Mixer [10] adapted to hold pint-sized Mason jars was used for suspending and mixing the material. The speed of the mixer was controlled by a variable resistance, set to deliver 20 to 25 volts. Aeration of the suspension was thus kept at a minimum. The suspension was transferred to 100 ml. glass centrifuge tubes.

Precipitation of the protamine-virus complex was allowed to take place at 0 to 8° C., for a period of 30 minutes, with frequent agitation of the centrifuge tubes. At the end of this period, P1 was centrifuged at

[4] The designation CT refers to experiment serial numbers of this laboratory.

[5] Hyaluronidase bovine testes, Worthington Biochemical Sales Co., Freehold, N. J.

[6] R.C.F. measurements at center of the tube.

[7] All recovered fractions were maintained at 0 to 8° C. where not otherwise indicated.

[8] Vacuum type Model SS-2. Manufactured by Ivan Sorvall, Inc., New York, N. Y.

[9] As already mentioned, the preparation of material T2 has been completely described in a thesis (*24*), and such material constituted standard lots of virus described previously (*3*). The preparation, indicated by the broken lines of the flow chart in text-figure 1, is as follows: The pellet (P1) is resuspended in an amount of 0.15 *M* potassium citrate to make the final concentration 1 gm. equivalent per ml. (*i.e.*, 1 ml. of suspension for every gram of tumor tissue processed). Suspension of the pellet is facilitated by use of a modified plastic Potter-Elvehjem homogenizer. The material is then twice cleared at 10,000 × g for 5 minutes in an International refrigerated centrifuge. Using a capillary-tipped pipette, the supernatant is recovered each time, with care not to disturb the loosely packed sediment.

[10] Ivan Sorvall, Inc., New York, N. Y.

2,300 × g for 20 minutes in the International refrigerated centrifuge. The supernatant from this run was poured off and discarded. The pellet P2 was resuspended in 100 ml. of 0.05 *M* sodium-citrate buffer, *p*H 7.5, containing trypsin[11] in the amount of 1.5 mg. per 100 ml. To facilitate suspension of the precipitated material, the Servall Omni-Mixer was used as above. Hydrolysis was carried out at room temperature for 1 hour. The suspension was cleared of large nondigested protamine-protein aggregates by centrifugation at 2,300 × g for 20 minutes as above. The supernatant S4 was poured off and ultracentrifuged at 40,000 × g for 1 hour in Spinco rotor No. 30[12] to sediment the highly active microsome fraction. Fraction P3, a glassy-appearing pellet, was resuspended in an amount of 0.05 *M* sodium-citrate buffer, *p*H 6.7, to give the desired concentration in terms of gram equivalents per ml. A modified plastic Potter-Elvehjem device was used to give an even suspension of the virus material. To assure a highly particulate suspension, free of large aggregates, fraction P3 was cleared two times at 10,000 × g for 5 minutes in the Spinco ultracentrifuge using rotor No. 40. The middle two-thirds portion of fraction S6, the final product, was removed with a capillary-tipped pipette.

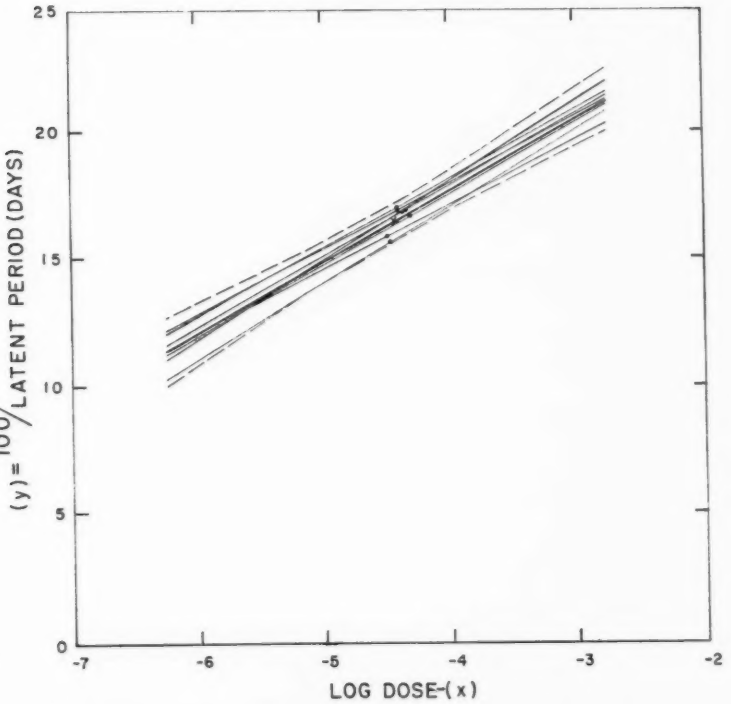
Standard virus preparation CT 629.—A study was undertaken to determine the biological reproducibility or variation among samples of a common lot of virus prepared as described above.

Under the designation CT 629, 35 gm. of tissue were processed to yield the highly active microsome fraction S6. In this experiment the concentration of the S6 fraction was 0.67 gm. equivalents per ml. The common lot (approximately 20 ml.) was sampled in 0.15 ml. aliquots and preserved as a standard virus preparation (see Materials and Methods). Seven of the frozen ampoules were then selected at random. Each was thawed and individually diluted in serial tenfold steps with citrate buffer containing hyaluronidase. Ten chickens were inoculated at each dose level, or a total of 80 chickens per sample.

Analysis of the data from the experiment was carried out as previously described (*3*). The results are shown graphically in text-figure 2. The closed circles (●), on the computed regression lines, represent the coordinate means ($\bar{x}$, $\bar{y}$) for each of the samples. The common regression line computed from all the data at each dose level is described by the heavy dark line. The curved broken lines represent the estimated limits of chance variation ($P=0.05$) of individual samples about the common regression line, computed from the mean number of individuals in a single sample. It is seen that variations between samples fall well within the limits of chance variation. Thus on the basis of biological response the samples of the common lot vary only within the limits of experimental error.

The results of preliminary investigation into the biochemical nature of the "partially purified" virus suspension CT 629 are shown in table 1. Of particular interest is the value given for total nitrogen, namely, 25.3 μg. per ml. This figure, when related to biological activity, serves as a

[11] Trypsin 2X crystalline containing 50 percent $MgSO_4$. Nutritional Biochemicals Corp., Cleveland, Ohio.
[12] Spinco ultracentrifuge, Model L, Specialized Instrument Corp., Belmont, Calif.



TEXT-FIGURE 2.—Composite chart describing regression lines of seven samples of a common lot of virus, standard preparation CT 629, in relation to a common regression line [*dark line;* (see text)].

measure of the degree of separation of nonactive nitrogenous material from the active virus particles. The value given here represents approximately a tenfold decrease in total nitrogen over standard preparations previously reported (*3*). Sixty-six percent of the total nitrogen content is accounted for as protein nitrogen.

With regard to the nucleic acid present in the standard preparation, no desoxypentosenucleic acid (DNA) was found, as measured by the Dische reaction. The absorption of the nitrogenous bases in the perchloric extracts of the samples was entirely accounted for by the amount of PNA

TABLE 1.—*Summary of results of chemical analyses of standard virus preparation CT 629*

Analysis	μg./ml.
Total P	6.3
Total N	25.3
Protein N	16.8
PNA	17.0
DNA	0.0

found to be present in the acid extract, as measured by the orcinol reaction. The inability to measure DNA in the extract of the standard preparation is an indication that the material is essentially free of contamination by nuclear constituents.

The value (6.3 μg./ml.) given for total phosphorus is a measure of the lipide, acid soluble-, nucleic acid-, and protein-phosphorus content of the purified microsome fraction S6.

Standard virus preparation CT 632.—Recently, work was undertaken in this laboratory to stockpile a large quantity of material S6 (see text-fig.1). A large pool of partially purified, highly active virus material would then be available for use as a standard of reference for quantitative investigations and would serve as a source material for further purification studies.

At intervals over a period of 5 months, twenty-three 35 gm. lots of certified bacteria-free tumor tissue were processed to yield the microsome fraction S6 in a final concentration of 1 gm. equivalent per ml. On the day each lot was completed it was divided into 10 ml. aliquots, each aliquot being sealed in pyrex glass tubes while under reduced pressure and quick-frozen in a mixture of dry ice and methyl cellosolve. The materials were stored in a frozen CO_2 box. At the end of the 5-month period, approximately 300 ml. (in the 10 ml. lots) were quickly thawed in a water bath (37° C.), pooled, and thoroughly mixed while maintained at a temperature of approximately 5° C. To insure the removal of any aggregated material which might have formed during the freezing and thawing process the pooled material was centrifuged at 2,300 × g for 10 minutes and the supernatant recovered.

From this fraction, approximately 1,000 ampoules, each containing 0.15 ml. of the virus suspension, were prepared as previously described (see Materials and Methods). The remaining supernatant was divided into 1, 3, 5, and 10 ml. lots, re-evacuated, sealed, and quick-frozen as above. All materials were again stored in a frozen CO_2 box.

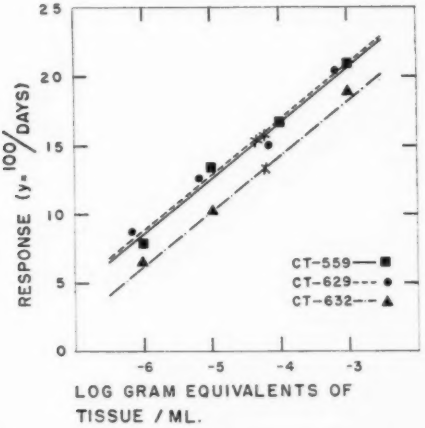
The results of a study similar to that described for CT 629, to test for biological reproducibility of the samples, showed no significant variation among the samples of the standard preparation CT 632.

Potency Relationships of Different Standard Preparations

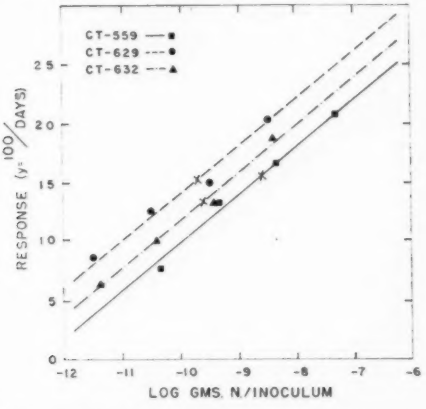
An experiment was designed to compare the relative biological activities of the recently prepared standards (CT 632 and CT 629) with an older standard preparation CT 559 [see reference (*3*) and footnote 9]. In this case, single ampoules of each of the above materials were selected at random, thawed, and appropriately diluted in serial tenfold steps. Twenty chickens were inoculated at each dose level of each material. The resulting data were analyzed by methods described elsewhere (*25*).

Text-figures 3 and 4 show the results of this experiment. In text-figure 3 response is plotted as a function of log-gram equivalents per ml. of virus materials, whereas in text-figure 4 chicken response is related to log grams of nitrogen in the inoculums (0.2 ml.). The regression lines drawn

through the dose-group means have a common slope determined from all the data. The crossed symbols (X) are the coordinate means ($\bar{x}$, $\bar{y}$) of the respective standard preparations.



TEXT-FIGURE 3.—Curves relating biological activity of three standard virus preparations on a gram-equivalent basis (see text).



TEXT-FIGURE 4.—Curves relating biological activity of three standard virus preparations on a total-nitrogen basis (see text).

In estimating the relative potencies of these three materials, the standard of reference used to establish the 100 percent potency level was material CT 559. The potencies of the other suspensions, CT 629 and CT 632, were then determined in relation to this level. The logarithms of the potency ratios were estimated from the horizontal distance between the respective dose-response curves (*26*).

When the biological activity of these materials is so determined and related on a gram-equivalent basis, as shown in table 2 and text-figure 3,

TABLE 2.—*Data showing relation of three standard virus preparations with respect to potency and nitrogen content*

Material (*expt. no.*)	Total nitrogen		Relative potency†				
			On gram-equivalent basis			On total-nitrogen basis	
	μg./ml. (*)	Percent (†)	Log percent	Standard error of log percent	Percent	Log percent	Percent
CT 559	250. 0	100. 0	2. 00	—	100. 0	2. 00	100. 0
CT 629	25. 3	10. 12	2. 09‡	±0. 12	124. 0	3. 09	1224. 0
CT 632	21. 2	8. 48	1. 44	±0. 12	27. 4	2. 51	323. 0

*Error of nitrogen determinations <5.0 percent.
†Referred to CT 559.
‡Corrected for original concentration of 0.67 gm. equivalents per ml.

it is seen that standard preparation CT 629 is more active than the reference material by 0.09 log intervals, whereas standard CT 632 is less active by 0.56 log intervals. The lower activity of CT 632 compared with CT 629 may possibly be explained by some denaturation of the agent during the additional steps of freezing and thawing of the suspension (S6) in 10 ml. volumes. (See standard virus preparation CT 632.)

When the relative potencies of the three preparations are compared on a total-nitrogen basis, as is seen in table 2, and the animal responses plotted as a function of nitrogen content, as in text-figure 4, it is seen that standard preparation CT 632 is three times as active as the reference material CT 559. Likewise, material CT 629 has over 12 times the biological activity of the older standard preparation. Therefore, although the total quantity of agent in CT 632 is somewhat diminished, the suspension has a sufficiently high level of activity per unit of nitrogen to warrant its use as a standard reference material and as a common source material for further purification and biochemical studies.

Discussion

The value of citrate salts in stabilizing the extracts of the Rous sarcoma (*1*, *2*) is further demonstrated in the extraction procedure described in this report.

Earlier experiments indicated that when crude microsome fractions, derived from different lots of Rous sarcoma tissue, are precipitated by protamine from a water solution, extreme variations occurred in the biological responses to the virus suspension prepared after further treatment. This source of variation was satisfactorily overcome by the use of citrate buffer, at a low molar concentration, as the protamine precipitating medium.

Kleczkowski (*27*) demonstrated that protamine-protein complexes are readily destroyed in 0.3 *M* NaCl solutions. In the case of the protamine-

Rous virus complex, mild hydrolysis by the proteolytic enzyme trypsin, in 0.05 *M* citrate buffer at a *p*H of 7.5, dissociates the protamine-virus protein complex formation. This disruption of the complex is without loss of activity of the virus, due possibly to a type of competitive enzyme inhibition where the protamine, rich in arginine, competes for the reactive centers of the enzyme leaving the virus to be recovered after further differential centrifugation.

The use of the basic protein protamine, then, affords a method for the separation and concentration of the active principle of the Rous sarcoma.

In considering the biological potencies of CT 629 and CT 632 relative to that of the earlier standard preparation CT 559, it should be emphasized here that a material (*3*) prepared in a way identical to CT 559 was found to be over 90 times more active, on a nitrogen basis, than fractions of the Rous sarcoma derived by earlier techniques of extraction, namely, that described as material CT 501 in a previous report (*3*). Standard virus preparations CT 629 and CT 632 are shown to be reproducible and to have more potency, per unit of nitrogen, than preparation CT 559. This high biological activity of the suspensions, associated with reproducibility and stability of the samples in citrate media, meets the criteria of materials suitable for use as standards of reference.

Summary

A procedure for the preparation of a partially purified suspension of the Rous sarcoma virus is presented. This is a physicochemical procedure involving the use of protamine, followed by proteolytic digestion and differential centrifugation. The resulting extract has sufficient biological activity to warrant use of the material as a standard of reference for quantitative biological studies and as a common source material for studies on further purification of the agent. The preparation of standard lots of virus and their biological activity and biochemistry are discussed.

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Chronic Gastritis of the Glandular Stomach, Adenomatous Polyps of the Duodenum, and Calcareous Pericarditis in Strain DBA Mice[1]

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The DBA strain of mouse is of particular interest because of the high incidence of a variety of unusual spontaneous lesions that develop in different organs and tissues in both sexes of these animals. Malignant lymphomas (*1*) are of common occurrence. Several lesions (*2*) of a degenerative nature develop in the pericardium, myocardium, skeletal muscle, subcutaneous adipose tissue, the cornea of the eye, and blood vessels. Female mice of this strain rather frequently develop cystic changes in the ovaries (*3*), adenomyosis of the corpus uteri, and carcinoma of the mammary glands. The present paper deals with the pathologic features of calcareous pericarditis, duodenal polyps, and chronic gastritis (*4,5*) observed in strain DBA mice and the influence of a stock diet and a low-roughage diet on the incidence of lesions in both sexes.

Materials and Methods

One hundred fifty strain DBA mice, 62 males and 88 females, were used in the first part of this study (expt. 260). Five to 10 mice of the same sex were caged together shortly after weaning. They were fed pellets of Purina dog chow which contains as much as 7 percent roughage and given tap water *ad libitum*. None of these mice were ever bred. Each month a few mice were killed and autopsied. The remainder, consisting of a larger number, were allowed to live out their full span of life or were killed only when death seemed imminent. The oldest male mouse died at 17 months of age, while the oldest female mouse died at 20 months of age.

In the second part of this study (expt. 315), 67 strain DBA mice, 32 males and 35 females, were handled under the same experimental conditions except that they were fed a low-roughage diet containing less than 1 percent roughage (tables 1 and 2).

Moribund animals were sacrificed and autopsied. This selection may have led to overstatement of the frequency of some of these conditions among younger mice.

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TABLE 1.—*Low-roughage diet*

Constituents	Amount in 6 kg. of mixture
Casein—washed from milk*	1,080 gm.
Corn starch†	3,150 gm.
Brewer's yeast—dry powder‡	480 gm.
"Swiftning" shortening from animal and vegetable fats§	900 gm.
Osborne-Mendel salt mixture—as modified by Haw, Oser, and Summerson (*6*)	240 gm.
Cod-liver oil—containing 2,000 U.S.P. units vitamin A per gm. and 125 U.S.P. units vitamin D per gm.‖	120 cc.
Wheat-germ oil (vitamin E)¶	30 cc.

*Fisher Scientific Co., St. Louis.
†Clinton Industries, Inc., Clinton, Ia.
‡Eimer and Amend, New York.
§Swift and Co., Chicago.
‖Maltine Co., New York.
¶Sharp & Dohme, Inc., Philadelphia.

DBA mice of subline 212 are used at the National Cancer Institute. The inbred strain DBA mice were originally derived from a single pair of mice obtained in 1909 by Little (*7*). To the best of our knowledge, strain DBA mice are not related to strain I mice (*8*). The reason for making this point is because strain I mice also develop spontaneous gastritis.

Necropsies were performed on all mice. At necropsy, in most cases, fixing fluid was injected into the stomach after the esophagus and duodenum were doubly ligated and cut between ligatures. The specimen was then dropped in fixative. After fixation, the stomach and duodenum were usually divided into an anterior and posterior half by a coronal section. In other cases, the stomach and duodenum were immediately opened at the necropsy, pinned to a cork board, and fixed. Zenker's

TABLE 2.—*Modified Osborne-Mendel salt mixture low-roughage diet* (6)

Constituents	Amount in 1 kg. of mixture (*gm.*)	
Calcium citrate $.4H_2O$		308. 35
$Ca(H_2PO_4)_2.H_2O$		112. 76
K_2HPO_4		218. 78
KCl		124. 76
NaCl		77. 08
$CaCO_3$		68. 60
$MgCO_3$		35. 17
$MgSO_4$ (anhydrous)		38. 34
Ferric citrate U.S.P. VIII	94. 81 gm.	
NaF	3. 13 "	
$MnSO_4.2H_2O$	1. 24	
$KAl(SO_4)_2.12H_2O$	0. 57	
KI	0. 25	
	100. 00 gm.	16. 16
		1, 000. 00

acetic-acid fluid was used for fixing some of the tissues from all the animals; some tissue was also fixed in neutral 4 percent aqueous formaldehyde solution. Paraffin sections of all tissues were stained with hematoxylin and eosin. Sections of all stomachs were also colored by the periodic-acid-Schiff method of McManus (*9*). Silver impregnation for reticulum, van Gieson's stain, and the phosphotungstic acid hematoxylin stains were used on occasion.

Chronic Gastritis

During life, distention of the stomach was sufficiently marked in a few mice to cause a slight visible prominence of the abdomen and increased rigidity of the abdominal muscles. However, in most mice, the stomach was not palpable clinically.

At necropsy the glandular stomach of many of the DBA mice, viewed from the serosal aspect, was found to be firm, rigid, and constricted, and the limiting ridge was deeply recessed. This chamber was readily distended upon the injection of fixing fluid, indicating that its previous condition of constriction was probably the result of a spastic state rather than fibrosis. Upon opening the glandular stomach prior to fixation, the rugae, particularly those in the proximal portion, were commonly found to be hypertrophied (fig. 1). In some mice the mucosa formed numerous small, round, gray elevations that extended from the limiting ridge to the pyloric ring (fig. 2). This pebbling of the mucosa (*pebblestone gastritis*) occurred independently or in combination with hypertrophic rugae (figs. 3 and 4) but usually preceded any other gross evidence of mucosal hypertrophy. In a few cases the lesions of hypertrophic gastritis were so severe (figs. 4 and 5) that there was pyloric obstruction with retention of food and gaseous dilatation of the stomach.

The forestomach showed hyperkeratosis in some cases, and this change was particularly marked at the limiting ridge (figs. 1 and 5). The presence of hyperkeratosis of the forestomach may reflect the inability of DBA mice to utilize biologically essential elements of the diet, the result of faulty metabolism secondary to gastritis.

The enlarged rugae of the fundic end of the glandular stomach were found to be caused by a thickening of the mucosa and by edema and an inflammatory cell infiltrate found chiefly in the submucosa (figs. 6 and 7). The inflammatory cells consisted of lymphocytes, plasma cells, and, less frequently, granulocytes. Impression smears of unfixed gastric mucosa revealed no consistent microbiological flora, although there were usually a large number of yeast forms and bacterial forms from the surface.

The thickened epithelium of the hypertrophied rugae of the glandular stomach showed a numerical increase of all the normal cellular elements. The greater the length of the mucosal glands, the greater the number of parietal, zymogen, and mucous cells (fig. 8). Some gastric glands penetrated the muscularis mucosae (figs. 9 and 10), but in no instance was there extension of epithelium beyond the confines of the submucosa.

The pebblestone lesion in the antral region of the glandular stomach

was found to consist of little knobs of thickened mucosal epithelium overlying an edematous lamina propria. The mucous cells were significantly increased in this lesion. Paneth's cells were occasionally found in the antral mucosa. This is a common finding in chronic atrophic gastritis in man.

Incidence.—The data on the mice with gastritis are shown in table 3 (see also table 6).

The incidence of the lesion of gastritis increased in both sexes with age, regardless of the amount of roughage in the diet. The percentages of mice with gastritis varied from 22 to 64 at 6 months of age or less and from 88 to 100 at 13 months of age or more. The difference in the incidence of gastritis in the male and female mice of the different groups was not marked except for the youngest age group and, with this exception, is probably not statistically significant. Twenty-six and seven-tenths percent of young mice fed the low-roughage diet developed gastritis, whereas the incidence of this lesion in young mice fed the high-roughage diet was 64 percent. There is no significant incidence difference in gastritis attributable to diet in the age groups 7 to 20 months.

Andervont (*10*) found that strain I mice fed food containing a large amount of roughage may develop more extensive lesions of gastritis than those fed a bland diet. In the present experiment, the low-roughage diet did influence the incidence of gastritis in strain DBA mice in the 1- to 6-month age group. In this young age group fewer mice ingesting the low-roughage diet had the lesion of gastritis than those fed the stock diet.

Duodenal Polyps

The duodenal polyps (figs. 2, 3, 4, 5, 11, 12, 13, and 14) were, as a rule, flat on the surface and broad at the base. Most often only 1 polyp was found in an animal, although 2, 3, 4, and occasionally as many as 7 polyps were counted in 1 animal. No lesion 1 mm. or less in diameter was listed as a polyp. Most of the polyps were located in the first portion of the duodenum, but they also occurred in the distal segment of the duodenum in a few mice. None of the polyps were found at the termination of the common bile duct in the limited number of mice in which the ampulla of Vater was identified at necropsy. In no case was there evidence of obstructive jaundice. A polyp commonly occupied as much as 50 percent of the lumen of the duodenum. The polyps did not show evidence of hemorrhage or ulceration.

Microscopically, the duodenal polyps were composed of hypertrophied duodenal mucosa with evidence of slight submucosal edema and a slight inflammatory cell infiltration (figs. 15 and 16). Many of the glands in the polyp were dilated and lined by hyperplastic epithelial cells. The number and distribution of mucous cells and of Paneth cells in the mucosa composing the polyps corresponded roughly with those of the normal mucosa of the duodenum. For that reason these polypoid lesions are considered to be focal overgrowths of mucosa with some dilatation of glands and hyperplasia of the epithelial cells rather than genuine neo-

TABLE 3.—*Incidence of lesions of gastritis in DBA mice of different age and sex fed different diets*

Experiment	Sex and number*		Age 1–6 months			Age 7–12 months			Age 13–20 months		
			No. in age group	No. with gastritis	Percent of mice with gastritis	No. in age group	No. with gastritis	Percent of mice with gastritis	No. in age group	No. with gastritis	Percent of mice with gastritis
#260 fed stock diet	Males	62	14	9	64. 3	31	25	80. 6	17	15	88. 2
	Females	88	11	7	63. 6	10	10	100. 0	67	66	98. 5
	Total	150	25	16	64. 0	41	35	85. 4	84	81	96. 4
#315 fed low-roughage diet	Males	31	9	2	22. 2	19	17	89. 5	3	3	100. 0
	Females	35	6	2	33. 3	22	18	81. 8	7	7	100. 0
	Total	66	15	4	26. 7	41	35	85. 4	10	10	100. 0

*Total number of male mice with gastritis: 71 of 93=76.3%.
Total number of female mice with gastritis: 110 of 123=89.4%.

plasms. Mitotic figures were not excessively numerous and they did not appear abnormal in form. No mitotically dividing cell could be identified as a Paneth cell.

At any given age, duodenal polyps occurred in animals fed the stock diet more frequently than in animals fed the low-roughage diet (table 4; see also table 6). While this difference is slight in the 7- to 12-month age group, it is pronounced in the 1- to 6-month and 13- to 20-month age groups.

There are some striking, but inconsistent and unexplainable, differences in the incidence of duodenal polyps in male and female mice. There is little sex difference in the incidence of duodenal polyps in either of the two age groups—1 to 6 months and 7 to 12 months—when the animals were fed the stock diet. However, at 13 to 20 months of age, the females showed an 86.6 percent incidence of polyps as compared to an incidence of only 29.4 percent in males. In the animals fed the low-roughage diet, there was an increased incidence of polyps in the females at 1 to 6 and 7 to 12 months of age as compared to the male mice of the corresponding age groups. There were too few males fed the low-roughage diet in the 13- to 20-month age group to permit conclusions to be drawn. However, only 1 of 7 females that ingested the low-roughage diet in the 13- to 20-month age group developed polyps. Thus the incidence of polyps was less in the oldest than in the two younger age groups of female mice fed the low-roughage diet, but this difference was not statistically significant.

Calcareous Pericarditis

White flecks were frequently found on the ventral surface of the heart in strain DBA mice at necropsy. These flecks were confluent in some cases and extended over the entire anterior surface of the heart. The smallest flecks could be scraped away with a knife. A distinct grating sensation was elicited upon lightly scraping the large flecks with a knife or needle. The posterior surface of the heart was only rarely involved by extension of this lesion from the anterior surface. The parietal pericardium usually showed little or no change. There was a small collection of fluid in the pericardial cavity in a few cases.

On microscopic examination the white flecks noted grossly were found to involve chiefly the pericardium. Occasionally the myocardium was involved superficially by this lesion and rarely in its entire thickness (figs. 17 and 18). The lesion consisted of degenerated and hyalinized connective tissue and myocardial fibers with focal and diffuse calcification. Some evidence of edema was often found around the hyalinized areas, and occasionally a focal infiltration of inflammatory cells in the myocardium was observed (fig. 19). Insofar as the pathogenesis of this process could be determined, there appeared to be primarily a degeneration of the local tissues and hyalinization of muscle fibers; this was followed by the formation of scar tissue which underwent a hyaline change; finally there was a deposition of calcareous material. Calcification appeared to occur in some cases without preceding hyalinization. Focal

TABLE 4.—*Incidence of duodenal polyps in DBA mice of different age and sex fed different diets*

Experiment	Sex	Age 1–6 months			Age 7–12 months			Age 13–20 months		
		No. in age group	No. with polyps	Percent with polyps	No. in age group	No. with polyps	Percent with polyps	No. in age group	No. with polyps	Percent with polyps
#260 fed stock diet	Males	14	4	28. 6	31	5	16. 1	17	5	29. 4
	Females	11	4	36. 4	10	1	10. 0	67	58	86. 6
	Total	25	8	32. 0	41	6	14. 6	84	63	75. 0
#315 fed low-roughage diet	Males	9	0	0. 0	19	0	0. 0	3	1	33. 3
	Females	6	1	16. 7	22	5	22. 7	7	1	14. 3
	Total	15	1	6. 7	41	5	12. 2	10	2	20. 0

TABLE 5.—*Incidence of lesions of pericarditis in DBA mice of different age and sex fed different diets*

Experiment	Sex	Age 1–6 months			Age 7–12 months			Age 13–20 months		
		No. in age group	No. with pericarditis	Percent with pericarditis	No. in age group	No. with pericarditis	Percent with pericarditis	No. in age group	No. with pericarditis	Percent with pericarditis
#260 fed stock diet	Males	14	8	57. 1	31	9	29. 0	17	6	35. 3
	Females	11	0	0. 0	10	5	50. 0	67	57	85. 1
	Total	25	8	32. 0	41	14	34. 1	84	63	75. 0
#315 fed low-rough-age diet	Males	9	2	22. 2	19	10	52. 6	3	2	66. 7
	Females	6	2	33. 3	22	8	36. 4	7	3	42. 9
	Total	15	4	26. 7	41	18	43. 9	10	5	50. 0

areas of hyalinization, calcification, or both were found in a few mice deep within the myocardium and even involving the endocardium. However, the most striking alteration was confined to the pericardium. A possible etiologic factor in the development of this lesion was nutritional deficiency secondary to the lesion of gastritis that invariably occurred in mice with the cardiac lesion. Cardiac lesions have been described in cases of nutritional deficiency (*11*).

Table 5 shows that the incidence of the lesion of pericarditis generally increased with the age of the mice (see also table 6).

Of the animals fed the stock diet, the incidence of calcareous pericarditis was higher in the males of the two younger age groups and higher in the females of the two older age groups. Perhaps the more susceptible male mice died of pericarditis at an earlier age than females, which developed this lesion at a later age than the males. At the beginning of experiment #260 there were 88 female mice and 62 male mice. Only 17 males, as compared with 67 females, lived more than 1 year. The cardiac lesion may have accounted in part for this difference in mortality between the sexes.

The incidence of the lesion of calcareous pericarditis in mice fed the low-roughage diet was higher, on the average, in males than in females. The mice ingesting the low-roughage diet did not appear to be as healthy and did not live as long as those fed the stock diet. Some of them died with diarrhea. The low-roughage diet was obviously not as satisfactory for mice as the stock diet.

Miscellaneous Lesions

In addition to the pathologic processes described in some detail in this communication, several other types of lesion were also observed. These included leukemia, *Klosiella muris* infection of the kidney, amyloid infiltration of various organs, an unidentified yellowish-brown pigment of the spleen, and focal hyperplasia, and in two cases, adenoma of the cortex of the adrenal gland. Urinary tract infections occasionally occurred in male mice. Female mice developed carcinoma of the mammary glands, adenomyosis of the corpus uteri, and cysts of the ovary. A hyaline change accompanied by the deposition of calcium in the walls of various blood channels was observed in different areas of the body. The vessels showing these changes often became thrombosed. These vascular changes occurred most frequently in the liver (fig. 20).

Comment

Opinions differ regarding the significance of the association of chronic gastritis and gastric cancer in man (*12*). Konjetzny (*13*) and Hurst (*14*) believed that carcinoma of the stomach originated in lesions of gastritis in most cases. On the other hand, Borrmann (*15*) almost never found gastritis in cases of early gastric carcinoma. He suggested that the inflammatory process, when it appears in the stomach later in the course

TABLE 6.—*Data on age of mice with gastritis, duodenal polyps, and pericarditis*

Experiment	Sex	All mice		Mice with gastritis			Mice with duodenal polyps			Mice with pericarditis		
		Median age (*months*)	Mean age (*months*)	Age lesion first appeared (*months*)	Med an age (*months*)	Mean age (*months*)	Age lesion first appeared (*months*)	Median age (*months*)	Mean age (*months*)	Age lesion first appeared (*months*)	Median age (*months*)	Mean age (*months*)
#260 fed stock diet	Males	8	9. 1	3	8	9. 4	3	9	9. 5	3	7	8. 2
	Females	15	13. 4	2	15	12. 8	1	15	15. 0	7	15	15. 2
	Total	13	11. 6		14	11. 5		15	14. 0		14	13. 3
#315 fed low-roughage diet	Males	9	8. 3	2	9	9. 5	13	13	13. 0	6	9	9. 8
	Females	10	9. 0	2	10	10. 2	2	11	10. 1	2	9	9. 6
	Total	9	8. 7		10	9. 9		11	10. 5		9	9. 7

of cancer, may be secondary to the neoplasm. Guiss and Stewart (*16*) found no anatomical association between the two lesions but pointed out that both occur at about the same period of life and that carcinoma may predispose to gastritis. Schindler (*12*) felt that the association between the two lesions was so striking that a common etiologic factor had to be presumed.

Lower animals rarely develop spontaneous gastritis except for certain strains of mice. This lesion regularly occurs in strain I mice (*17,18*), Strong's strain NHO mice (*19*), and strain DBA mice (*5*). The lesion of gastritis has been induced experimentally in Macaca monkeys by infestation with the nematode *Nochtia nochti* (*20*); it has been induced in rats by infestation with *Cysticercus fasciolaris* (*Taenia crassicollis*) (*21, 22*) and in monkeys by the oral administration of diesel fuel oil (*23*). The spontaneous gastritis that occurs in strain DBA mice was first observed in an experiment (*4*) in which the carcinogen 20-methylcholanthrene was injected into the wall of the glandular stomach of mice of this strain. Gastritis was found to occur in the mice treated with the carcinogen and also in the untreated control mice. Despite the spontaneous development of chronic gastritis, mice of the DBA strain were found to be considerably more resistant to the experimental induction of gastric carcinoma by 20-methylcholanthrene than were the mice of the five other strains similarly tested (*4*). The mice of these other five strains did not exhibit the lesion of spontaneous gastritis. The lesion of chronic gastritis found in strain DBA mice is of the hypertrophic type and, therefore, not strictly comparable to the lesion of atrophic gastritis found in man in association with gastric cancer. It resembles more closely the lesion of giant hypertrophy of gastric mucosa described in patients by Kenney *et al.* (*24*). Whatever may be the significance of the association of these neoplastic and non-neoplastic gastric lesions in man, gastritis in lower animals has not been shown to be a predisposing factor for the development of gastric cancer either when it is induced experimentally or in the rare instances in which this neoplastic lesion occurs spontaneously.

The calcareous lesions involving the heart of strain DBA mice, the thrombotic lesions of the blood vessels, and other degenerative lesions of the myocardium, subcutaneous adipose tissue, and eye may possibly all have a common etiology. Maybe they represent a response to malnutrition that could result from faulty metabolism secondary to the gastritis and duodenal polyps. No physiological or nutritional studies have been carried out to determine the functional metabolic alterations occasioned by the gastritis and duodenal polyps in DBA mice, but it is safe to assume, on the basis of the pathologic findings, that alimentation is impaired to some extent. Whether the lesions in other body tissues are related to the gastrointestinal lesions requires further investigation.

Lesions of the heart identical with those of DBA mice have not been described in mice of other strains. Spontaneous focal myocarditis and endocarditis sometimes associated with pancarditis and vascular lesions were described in Swiss mice by Lenke and Loewe (*25*) and Gray (*26*).

These lesions in the Swiss mice had a larger inflammatory component and their distribution was different from those in the DBA mice. The lesions of the Swiss mice did not involve the pericardium so predominantly and did not show calcareous deposits to the same extent as those of the DBA mice. According to Dessau, Lipchuck, and Klein (*27*), absence of vitamins K and E from the diet of mice (strain not stated) was found to cause hemorrhagic and inflammatory lesions of the heart that healed by fibrosis and calcification. White and Mider (*28*) reported degenerative arterial changes with associated calcification of the large arteries of strain DBA mice when the skin of these mice, which were fed a diet low in cystine and methionine, was painted with 20-methylcholanthrene. The arterial lesions failed to develop in painted DBA mice when their diet was supplemented with cystine. The etiology of the spontaneous cardiovascular lesions in DBA mice described in the present communication is not known, although, as stated, it may possibly be due to a nutritional disturbance secondary to the gastrointestinal lesion.

The duodenal polyps that occur in mice of strain DBA are probably not genuine neoplasms since they are composed of a mixture of cell types that normally populate the mucosa. These polyps show only a limited capacity for growth. The presence of an inflammatory-cell infiltration in the stroma of the polyps is consistent with the concept that they represent a form of inflammatory adenomatoid hyperplasia. Such polypoid lesions occur in the upper respiratory passages and in the colon of man at the sites of chronic inflammation. The fact that the duodenal polyps of DBA mice increased in incidence when the mice were fed the high-roughage diet is to some extent corroborative evidence for this point of view.

The regular occurrence of a variety of degenerative lesions in the heart, skeletal muscle, subcutaneous fat, and eye of the DBA mice, as well as hyperplastic and hypertrophic lesions in the glandular stomach, duodenum, and uterus and neoplasms of breast and bone marrow, affords biologic material of some importance for investigation.

Summary

1) One hundred fifty strain DBA mice, 62 males and 88 females (expt. 260), were fed pellets of Purina dog chow containing as much as 7 percent roughage. Sixty-seven additional strain DBA mice, 32 males and 35 females (expt. 315), were fed a low-roughage diet containing less than 1 percent roughage. A few mice of both groups were killed and necropsied each month, and the remaining mice were allowed to live their full life span or until they appeared to be *in extremis*. To evaluate the data, the necropsied mice were divided into the following three age groups: 1 to 6 months, 7 to 12 months, and 13 to 20 months.

2) The lesions of chronic hypertrophic gastritis, duodenal polyps, and calcareous pericarditis were frequently observed, and their pathologic features are described. The incidence of all three types of lesions increased with the age of the mice.

The following lesions were also observed: malignant lymphoma; degenerative processes in the myocardium, skeletal muscle, subcutaneous adipose

tissue, cornea of the eye, and blood vessels; cystic ovaries; adenomyosis uteri; and mammary carcinoma.

3) The lesions of chronic hypertrophic gastritis and adenomatous polyps of the duodenum are not precancerous.

4) Young mice fed a low-roughage diet seemed to develop gastritis less frequently than young mice fed the stock diet. However, in time, mice of the two experimental groups showed no difference in the incidence of this lesion.

5) The incidence of duodenal polyps was lower in mice fed the low-roughage diet than in mice fed the stock diet.

6) The incidence of calcareous pericarditis was higher in male mice of the younger age group fed the stock diet and higher in the female mice in the older age group fed the same diet than in the animals of comparable age and sex fed the low-roughage diet.

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PLATE 73

FIGURE 1.—Stomach and duodenum. Note hypertrophied rugae in glandular stomach, hyperkeratosis of forestomach, and hypertrophy of limiting ridge. The stomach was opened along greater curvature, pinned to cork board, and then fixed. × 2

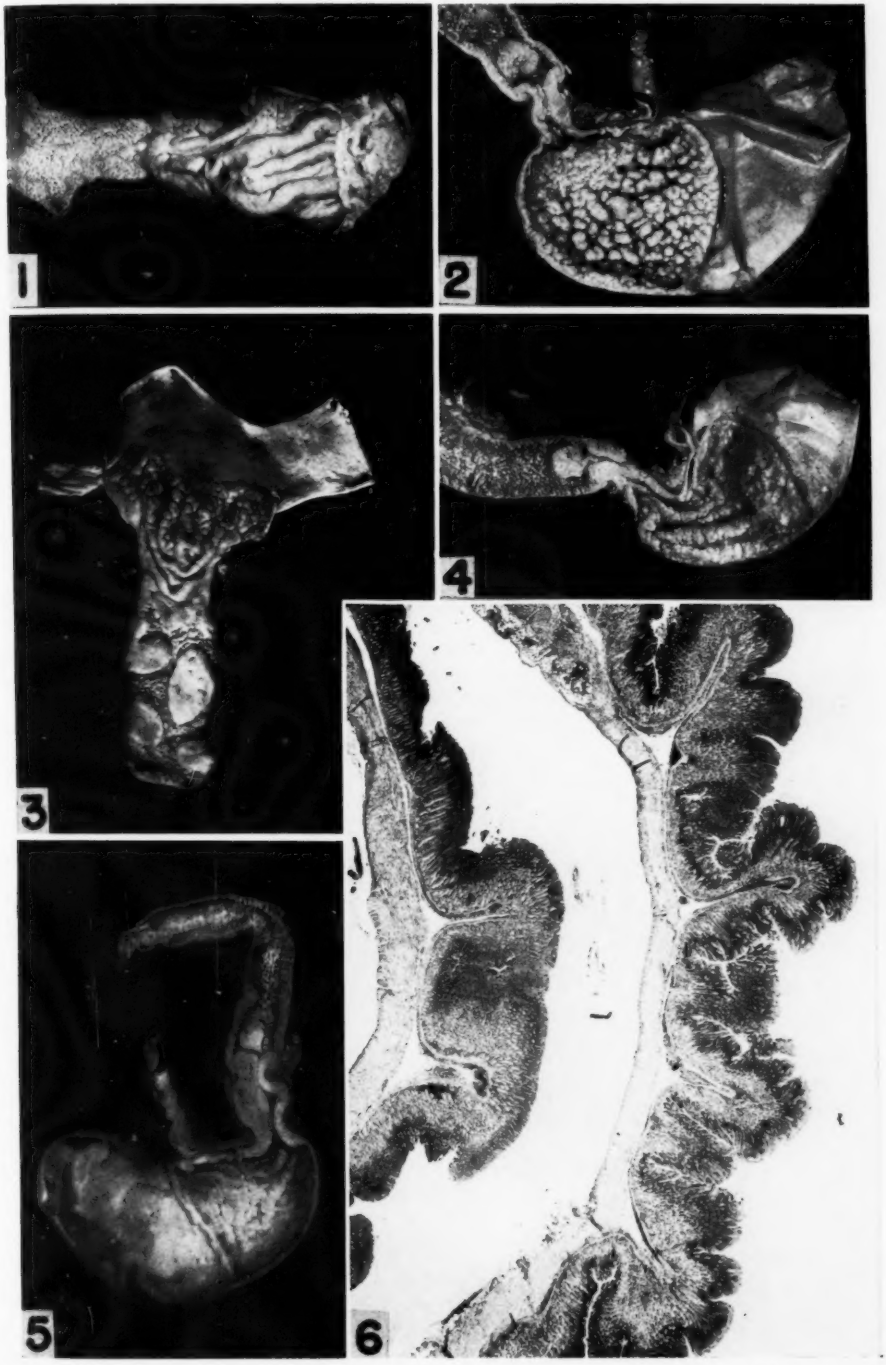
FIGURE 2.—Coronal section of stomach and duodenum. Note pebblestone hypertrophy of glandular mucosa and thickening and narrowing of antrum. There is a large, solitary, sessile duodenal polyp. Fixing fluid was injected into the lumen of the stomach at autopsy. × 2

FIGURE 3.—Stomach, opened along lesser curvature, and duodenum. The rugae are hypertrophied and the mucosa shows pebbling. Four large duodenal polyps are present. The specimen was pinned to a cork board and fixed. × 2

FIGURE 4.—Stomach and duodenum. There is stenosis of the antrum due to hypertrophy of the mucosa. The mucosa of the remainder of the glandular stomach is pebbled and hypertrophied. There is one duodenal polyp. Fixing fluid was injected into the lumen of the stomach at autopsy. × 2

FIGURE 5.—Stomach and duodenum. There is a single duodenal polyp. The antrum is partially obstructed due to thickening of the mucosa. The remainder of the glandular stomach shows the changes of hypertrophic gastritis. There is hyperkeratosis of the forestomach and hypertrophy of the limiting ridge. × 2

FIGURE 6.—Glandular stomach. The plane of the section is at right angles to the long axis of the hypertrophied rugae. There are focal collections of inflammatory cells in the different coats of the viscus. Note edema of the submucosa. Hematoxylin and eosin. Photomicrograph × 15

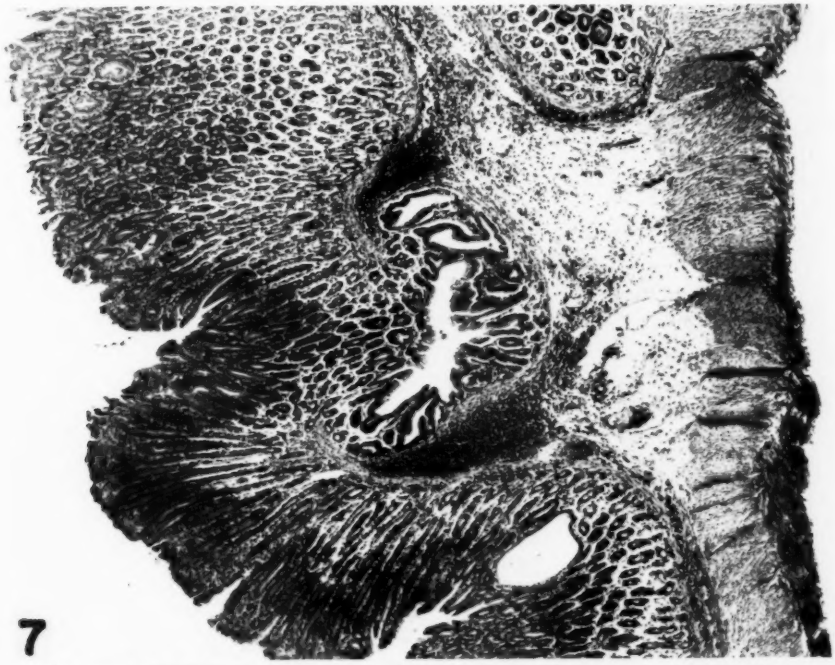


366462—56——10

PLATE 74

FIGURE 7.—Glandular stomach. Area of hypertrophic gastritis showing enlarged rugae with microcyst in mucosa. A focus of atypical glands lies deep in the mucosa and is separated from the submucosa by the attenuated muscularis mucosae. Note also the submucosal edema and inflammatory cells. Hematoxylin and eosin. Photomicrograph × 70

FIGURE 8.—Glandular stomach. This is a higher magnification of the field showing downward extension of the gastric glands illustrated in figure 7. Note parietal and zymogen cells and other normal components of the gastric mucosa. Hematoxylin and eosin. Photomicrograph × 440



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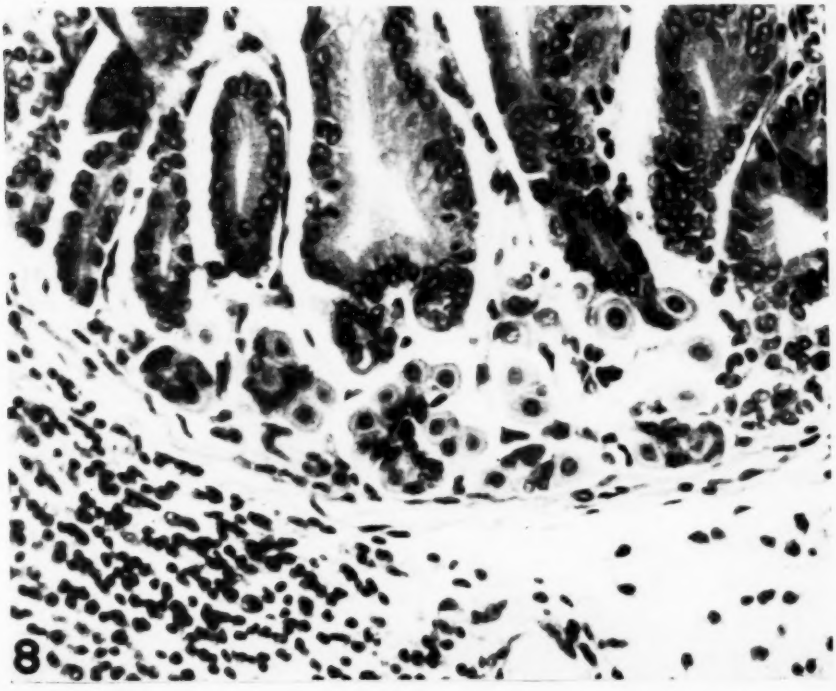

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PLATE 75

FIGURE 9.—Glandular stomach. An area of gastritis showing a focus of mucosal glands that have broken through the muscularis mucosae and are present in the submucosa. Hematoxylin and eosin. Photomicrograph × 33

FIGURE 10.—Glandular stomach. A lateral margin of the focus of glands within the submucosa shown in figure 9. The glands are irregular in form and the lining cells are somewhat atypical in appearance. A few mitotic figures are present. Hematoxylin and eosin. Photomicrograph × 400

FIGURE 11.—Duodenum. Sessile polyp. Microcysts like those in the illustration are commonly found in this type of lesion. Hematoxylin and eosin. Photomicrograph × 32

FIGURE 12.—Duodenum. Another polyp showing Brunner-type glands underlying the hyperplastic overgrowth of mucosa. A portion of normal duodenum may be seen at the right of the illustration. Hematoxylin and eosin. Photomicrograph × 33

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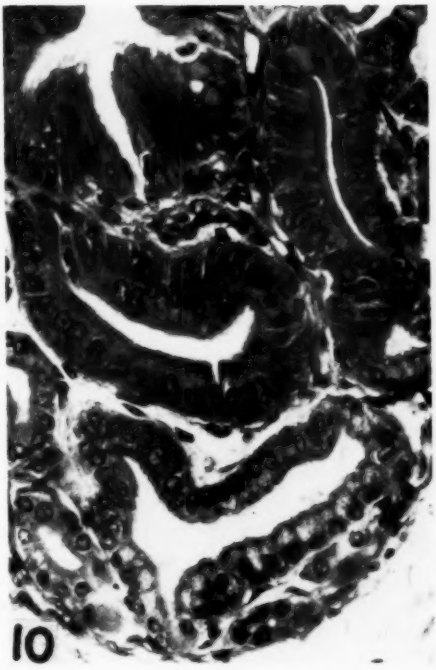

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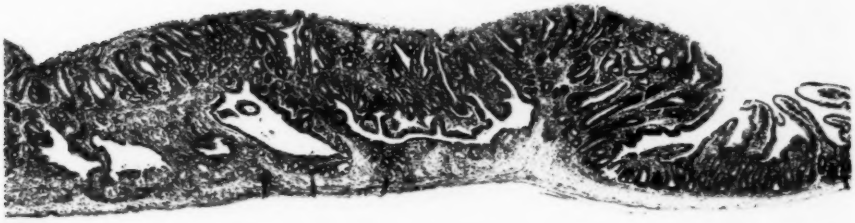

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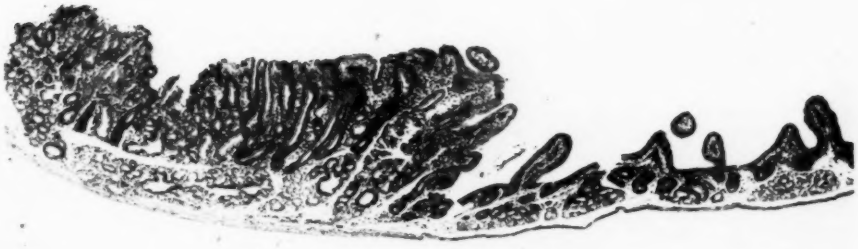

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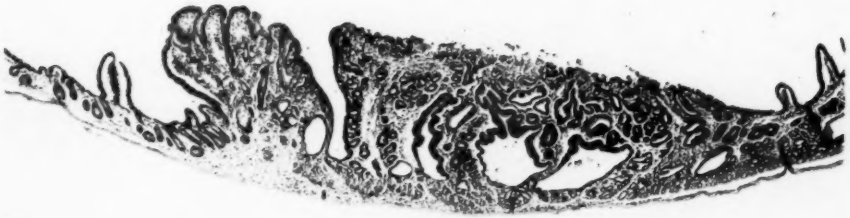
PLATE 76

FIGURE 13.—Duodenum. Two adjacent but separate polyps with several microcysts. Hematoxylin and eosin. Photomicrograph × 32

FIGURE 14.—Duodenum. A less common type of polyp showing a narrow base and stalk. Also note inflammatory cells in the stroma of the polyp. Hematoxylin and eosin. Photomicrograph × 33

FIGURE 15.—Duodenum. Higher magnification of polyp illustrated in figure 13 showing the irregularity in size and shape of the glands. At the base of the polyp there are Brunner-type glands. Note the inflammatory cells in the lamina propria. Hematoxylin and eosin. Photomicrograph × 100

FIGURE 16.—Duodenum. A still higher magnification of the polyp illustrated in figures 13 and 15, to show cytologic detail. There is a wide range of difference in the size of the cells lining the glands. The nuclei are hyperchromatic but are usually basally situated except where the cells are stratified. Hematoxylin and eosin. Photomicrograph × 400



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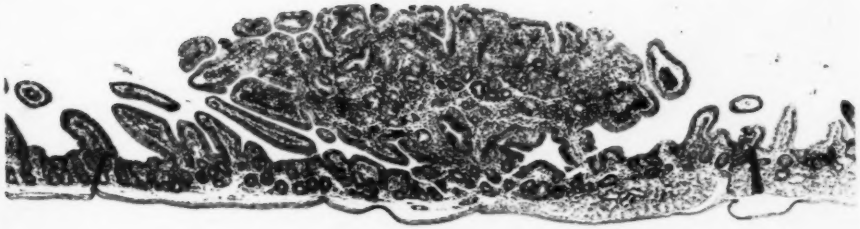

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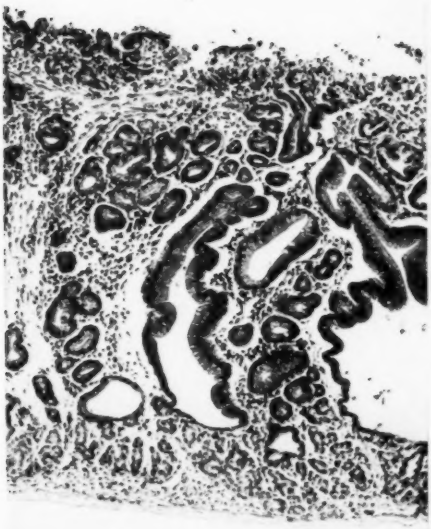

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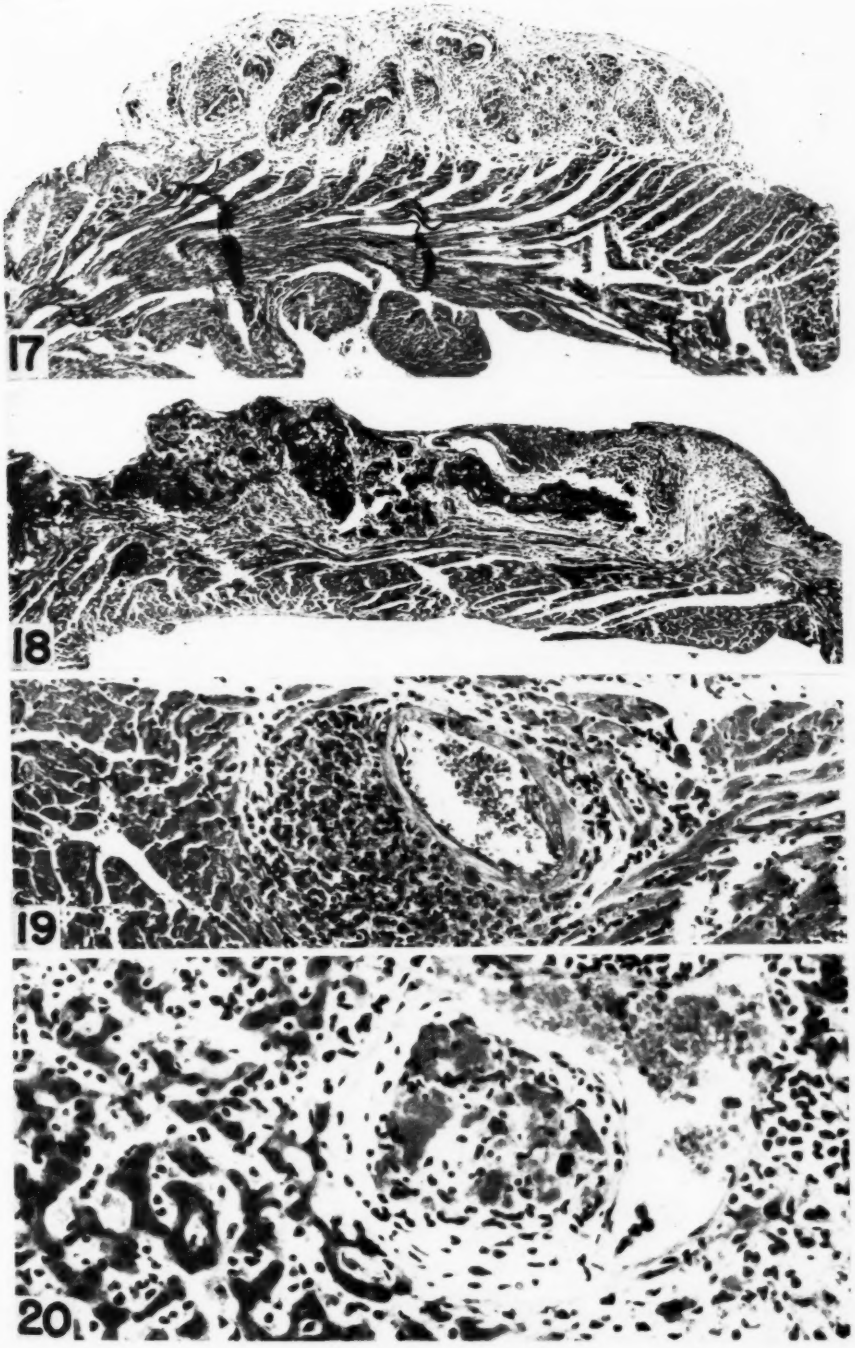
PLATE 77

FIGURE 17.—Heart. The fibrous, inflammatory tissue and calcareous deposits form a nodular mass that is elevated on the surface of the heart and does not involve the myocardium to the extent shown in figure 18. Hematoxylin and eosin. Photomicrograph × 60

FIGURE 18.—Heart. Chronic pericarditis and myocarditis with calcareous deposits. The lesion extends deeply into the muscle of the ventricle. Hematoxylin and eosin. Photomicrograph × 80

FIGURE 19.—Perivascular focus of inflammatory cells in myocardium. There is a small deposit of thrombotic material on the intima of the vessel. Hematoxylin and eosin. Photomicrograph × 210

FIGURE 20.—Liver. Organizing thrombus in intrahepatic branch of portal vein. Hematoxylin and eosin. Photomicrograph × 290



366462—56——11

Unusual Hydrolipotropic Shifts in the Spleen of Albino Rats Bearing Walker Carcinosarcoma 256[1, 2, 3]

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The transplantable tumor Walker carcinosarcoma 256 contains high concentrations of water, phospholipide, total cholesterol and free cholesterol, and low concentrations of neutral fat, per unit dry weight, throughout the period of its growth in albino rats (*1*). The homogenized carcass of non-tumor-bearing albino rats has a lower concentration of water, phospholipide, total cholesterol and free cholesterol, and a higher concentration of neutral fat per unit dry weight (*2*). As Walker carcinosarcoma 256 grows, the water level, the phospholipide concentration, and the concentration of total cholesterol and free cholesterol in the host rat increase and the level of neutral fat declines (*3*). These shifts in the concentration of water and lipides in the host rat are toward the concentration in the tumor; the term "hydrolipotropic shift" has been used to describe these phenomena (*3*). The hydrolipotropic shift in the homogenized carcass of albino rats betokens an influence of the tumor upon the host carcass. The investigation described herein was undertaken to determine the nature of the hydrolipotropic shift, if present, in the enlarged spleen of albino rats bearing Walker carcinosarcoma 256.

Littermate Analysis

Inoculation of Walker carcinosarcoma 256 was made into the groin of one of each of 56 pairs of inbred, albino rats (Wistar strain) that were litter mates by a method previously described by Boyd and McEwen (*1*). Thirty-six pairs were males and 20 pairs were females. Use of inbred litter mates practically eliminates variation due to genetic differences, as noted by Russell (*4*). The animals were fed Purina Fox Chow Checkers and water *ad libitum*. Each pair in the entire series (tumor-bearing rat and non-tumor-bearing litter mate) was sacrificed with chloroform on the same day after 14 to 34 (mean 24) days of tumor growth, at which time the age of the rats was 47 to 97 (mean 68) days.

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[3] A report of this investigation was presented before the Cancer Section of The Canadian Physiological Society, Annual Meeting, London, Ontario, October, 1955.

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The animals were weighed at the time of inoculation and at the time they were sacrificed. A summary of the values for body weight is given in table 1. The final body weight of the tumor-bearing rats was significantly greater than that of the non-tumor-bearing litter mates by a mean of 18 percent. From figures given by Boyd and McEwen (*1*) and by Boyd, Connell, and McEwen (*2*) the increase in body weight may be calculated to have been due to an increased amount of water in the tumor-bearing rats.

TABLE 1.—*Body weight of tumor-bearing albino rats and non-tumor-bearing litter mates*

	Initial weight $\overline{X} \pm \hat{\sigma}_{\bar{x}}$ (*gm.*)	Final weight $\overline{X} \pm \hat{\sigma}_{\bar{x}}$ (*gm.*)
Tumor-bearing rats	126 ± 5.14	233 ± 9.52
Non-tumor-bearing rats	124 ± 5.30	198 ± 6.61
Difference	2	35
P, difference=0	0.8	0.005

The tumors, primary and secondary (when present), were removed and weighed. A measure of the size of the tumor was made by calculating the T/RC coefficient as described by Boyd *et al.* (*3*). The T/RC coefficient is the wet weight of the tumor, in grams, divided by the wet weight of the residual carcass, in grams, and the quotient multiplied by 100. A summary of these data is presented in table 2. The statistical methods used in analyzing these and other data of this report and the statistical symbols are those described by Hill (*5*) and Croxton (*6*).

TABLE 2.—*Weight of excised tumors*

	Mean ± standard error ($\overline{X} \pm \hat{\sigma}_{\bar{x}}$)
Weight of tumor, gm	73.9±7.00
Weight of residual carcass, gm	159 ±5.24
T/RC coefficient	46.9±4.08

The spleen was removed and weighed, and aliquots were selected for determination of water and lipides, the latter by an adaptation of the oxidative micromethods of Bloor described by Boyd *et al.* (*7*). Results were calculated as gm. per 100 gm. dry weight of spleen, as mg. per spleen per kg. total body (tumor plus host) weight, and as mg. per spleen per kg. host weight, as indicated below.

Splenomegaly

The weight of the spleen of tumor-bearing rats increased significantly to about 3 times that in the non-tumor-bearing litter mates as shown by results summarized in table 3. Calculated as percent of total body

Vol. 16, No. 4, February 1956

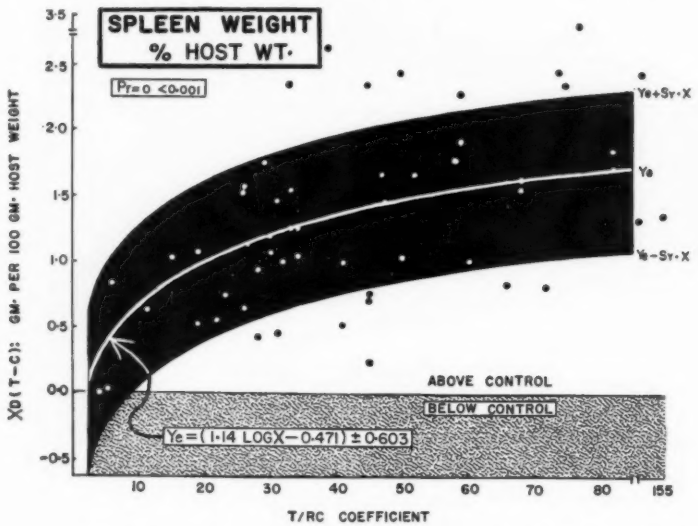
TABLE 3.—*Mean changes in the weight of, and concentration of water and lipides in, the spleen of albino rats bearing Walker carcinosarcoma 256*

	Number of pairs	$\overline{X}_C \pm \hat{\sigma}\,\overline{x}c$	$\overline{X}\ D(T-C) \pm \hat{\sigma}\,\overline{x}d(t-c)$	$P_{\overline{X}\ D(T-C)}$	$\frac{\overline{X}\ D(T-C)}{\overline{X}_C} \times 100$
Weight, gm	54	0. 900 ± 0. 035	1. 82 ± 0. 15	<0. 001	+202
Weight, % total body wt	54	0. 479 ± 0. 024	0. 757 ± 0. 057	<0. 001	+158
Weight, % host wt	54	0. 479 ± 0. 024	1. 315 ± 0. 097	<0. 001	+275
Water, % wet wt	54	77. 1 ± 0. 24	1. 94 ± 0. 22	<0. 001	+ 2. 52
Water, % dry wt	54	341 ± 4. 5	38. 1 ± 4. 9	<0. 001	+ 11. 2
Total lipide, % dry wt	47	7. 88 ± 0. 40	−0. 581 ± 0. 41	0. 2	− 7. 37
Neutral fat, % dry wt	47	1. 19 ± 0. 25	0. 196 ± 0. 26	0. 5	+ 16. 5
Total fatty acids, % dry wt	51	4. 78 ± 0. 29	−0. 031 ± 0. 27	0. 9	− 0. 7
Total cholesterol, % dry wt	52	1. 22 ± 0. 070	−0. 341 ± 0. 064	<0. 001	− 28. 0
Ester cholesterol, % dry wt	50	0. 0212 ± 0. 045	−0. 0452 ± 0. 060	0. 5	−213
Free cholesterol, % dry wt	51	1. 18 ± 0. 042	−0. 305 ± 0. 044	<0. 001	− 25. 8
Phospholipide, % dry wt	49	5. 44 ± 0. 28	−0. 293 ± 0. 264	0. 3	− 5. 43

weight, the proportion of spleen increased by a mean of 158 percent, while calculated as percent of residual carcass weight, the proportion of spleen increased by a mean of approximately 275 percent.

Changes in the weight of the spleen in tumor-bearing rats and in the proportion of the spleen to total body weight and host weight were correlated with the T/RC coefficient. The value of each measurement in a non-tumor-bearing rat (X_C) was subtracted from the corresponding value in its tumor-bearing litter mate (X_T) to obtain a difference [$\overline{X}\ D(T-C)$]. P values obtained by application of a t test to mean differences [$\overline{X}\ D(T-C)$] are listed in table 3. These $D(T-C)$ figures were then plotted against the value of the T/RC coefficient in the tumor-bearing rat. A scatter diagram of such a plot of spleen weight as percent of host weight is shown in text-figure 1. The two unknowns in the estimating equation were determined by the method of least squares to obtain an estimate of $Y(Ye)$. The estimated values of Y, together with *Ye plus* ($Ye+S_{Y.X}$) and *minus* ($Ye-S_{Y.X}$) the standard error of the estimates, have been plotted as regression lines in text-figure 1. The coefficient of correlation (r) was calculated by Pearson's product-moment formula to be $+0.485$. A t test indicated that the probability of r being 0 was highly unlikely ($Pr=o$ <0.001). The corresponding r for the proportion of spleen weight as percent of body (tumor+host) weight was $+0.173$ with a $Pr=o$ of 0.2. The corresponding calculations for weight of spleen in gm. wet weight were $r=+0.399$ and $Pr=o=0.005$, and a scatter diagram showed positive correlation with log T/RC coefficient.

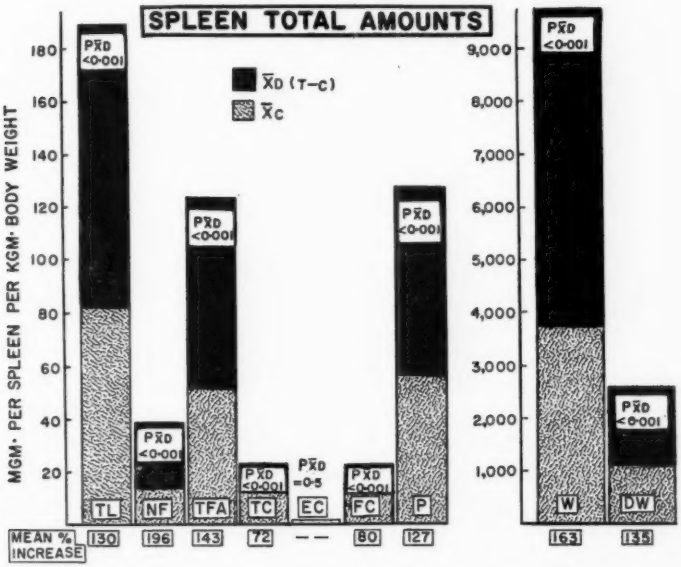
Therefore, the spleen increased in weight in tumor-bearing rats in linear correlation with the log of the T/RC coefficient. The correlation



TEXT-FIGURE 1.—Correlation, with the T/RC coefficient, of $D(T-C)$ values for weight of spleen as percent of host weight.

was more pronounced when weight of spleen was calculated as a percent of host weight and less pronounced, to the point of having a P value greater than 0.05, when calculated as percent of total body (tumor+host) weight.

Associated with increase in weight of the spleen were increases in the total amount of lipides contained in the organ. The results are summarized as bar diagrams in text-figure 2. The increase for water and neutral fat was above and the increase for total cholesterol and free cholesterol was below the range of increase of the remaining constituents. These results suggested that the concentration of cholesterol, expressed as gm. per 100 gm. dry weight, had decreased while that of water and neutral fat had increased in the spleen of tumor-bearing rats.



TEXT-FIGURE 2.—The mean total amount of lipides, water, and dry weight in the spleen of tumor-bearing albino rats [$\bar{X}\ D(T-C) + \bar{X}_C$] and non-tumor-bearing litter mates ($\bar{X}_C$). Legend: TL—total lipide; NF—neutral fat; TFA—total fatty acids; TC—total cholesterol; EC—ester cholesterol; FC—free cholesterol; P—phospholipide; W—water; DW—dry weight.

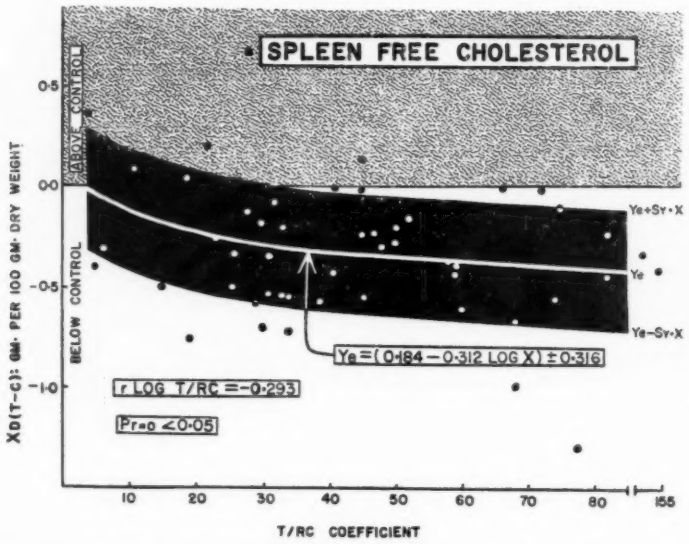
The Hydrolipotropic Shift

Data on the concentration of lipides, in gm. per 100 gm. dry weight, have been summarized in table 3. The values in non-tumor-bearing rats are of about the same order as corresponding levels in other species reviewed by Bloor (*8*) and similar to those in male rats reviewed by Deuel (*9*). The cholesterol concentrations were significantly lower in the tumor-bearing rats than in their non-tumor-bearing litter mates. This shift was in the opposite direction, away from the higher level in the tumor, and will be discussed below.

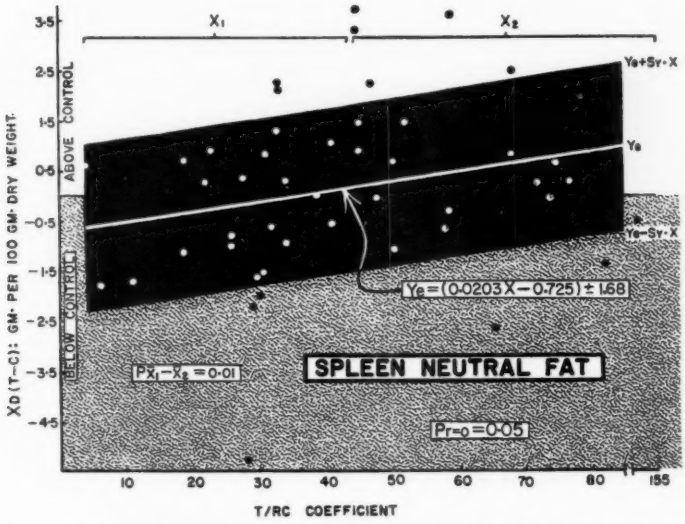
The concentration of free cholesterol in each tumor-bearing rat was subtracted from the concentration in its non-tumor-bearing litter mate. The differences [X $D(T-C)$] were then plotted against the log of the T/RC coefficients. The results are charted in text-figure 3. An estimating equation for linear correlation with log T/RC coefficient was found to fit the differences. The solution of this equation by the method of least squares is shown as a regression line plus and minus the standard error of the estimated value of Y in text-figure 3. The coefficient of correlation by Pearson's product-moment formula was calculated to be -0.293 and the probability of this being a zero correlation by the t test was <0.05.

These results indicated that the $D(T-C)$ concentration of free cholesterol in the spleen decreased in linear correlation with increasing log of the T/RC coefficient. There was no significant correlation between the $D(T-C)$ values for ester cholesterol levels and the T/RC coefficient ($r= +0.235$, $P=0.1$). The effect was to reduce to insignificance the correlation of $D(T-C)$ values for concentration of total cholesterol and the T/RC coefficient.

The $D(T-C)$ values for concentration of the remaining lipides were similarly plotted against the T/RC coefficient. Significant ($P=0.05$ or less) positive correlation with the value of the T/RC coefficient was found in the instances of total lipide, neutral fat, and total fatty acids, and no correlation in the instance of phospholipide. The significant positive correlation of the first three lipides just noted was due largely to shifts in the concentration of neutral fat in the spleen of the tumor-bearing rats.



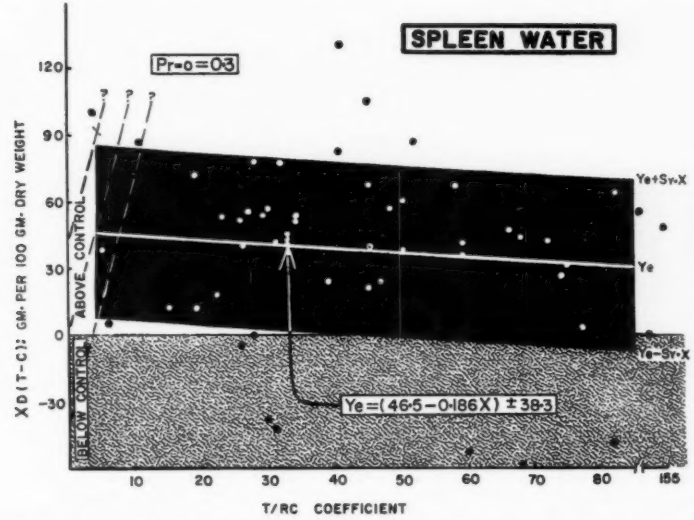
TEXT-FIGURE 3.—The $D(T-C)$ values for concentration of free cholesterol in the spleen correlated with the T/RC coefficient.



TEXT-FIGURE 4.—The $D(T-C)$ values for concentration of neutral fat in the spleen correlated with the T/RC coefficient.

The $D(T-C)$ values for concentration of neutral fat have been plotted against the T/RC coefficient in text-figure 4.

The $D(T-C)$ values for concentration of water were plotted against the T/RC coefficient in a corresponding manner, and the results are shown in text-figure 5. Even in animals bearing the smallest tumors



TEXT-FIGURE 5.—The $D(T-C)$ values for concentration of water in the spleen correlated with the T/RC coefficient.

Table 4.—*A comparison of measurements of the spleen in male and female albino rats*

Measurement	$\overline{X}$ $D(T-C)$			Correlation of X $D(T-C)$ with T/RC coefficient			
				Males		Females	
	Males (M)	Females (F)	P_{M-F}	r	$P_{r=o}$	r	$P_{r=o}$
Weight, % total body wt	+0. 802	+0. 677	0. 25	+0. 123	0. 5	+0. 267	0. 2
Weight, % host wt	+1. 38	+1. 21	0. 4	+0. 477	0. 005	+0. 513	0. 02
Water, % dry wt	+39. 4	+36. 0	0. 5	−0. 170	0. 3	−0. 088	0. 7
Total lipide, % dry wt	−0. 281	−0. 985	0. 4	−0. 023	0. 9	+0. 563	0. 01
Neutral fat, % dry wt	+0. 441	−0. 135	0. 3	+0. 083	0. 7	+0. 502	0. 02
Total fatty acids, % dry wt	+0. 216	−0. 415	0. 3	−0. 119	0. 5	+0. 523	0. 02
Total cholesterol, % dry wt	−0. 347	−0. 331	0. 9	−0. 032	0. 9	0. 00	1. 0
Ester cholesterol, % dry wt	−0. 020	−0. 083	0. 6	+0. 181	0. 3	+0. 405	0. 1
Free cholesterol, % dry wt	−0. 341	−0. 249	0. 4	−0. 262	0. 2	−0. 242	0. 3
Phospholipide, % dry wt	+0. 012	−0. 737	0. 2	−0. 345	0. 05	+0. 244	0. 3

found, the water levels in the spleen were elevated. The increase presumably started to appear in animals bearing still smaller tumors, and this assumption is indicated by interrupted lines and question marks in text-figure 5. No further significant increase or decrease in water levels occurred during the subsequent growth of the tumor. Increase in water levels of the spleen was an indication of an extremely early effect of Walker carcinosarcoma 256 upon the host rat.

Influence of Sex

The sex of the tumor-bearing rat was found to have no influence upon the shifts in concentration of water and cholesterol. Increases in the concentration of neutral fat with increasing T/RC coefficients tended to be more evident in females. The correlation coefficient between concentration of phospholipide and the T/RC coefficient differed significantly with sex. These data are summarized in table 4. Male rats gained body weight faster than did females. The tumor-bearing males gained more body weight than did the tumor-bearing females. However, the augmentation of body-weight gain by the tumor was not significantly greater in males than in females, at least not in calculations based upon the number of rats available. The spleen made up a significantly larger proportion of body weight in non-tumor-bearing female rats than in non-tumor-bearing males; this sex difference was not present in the tumor-bearing animals.

Discussion

The mass of sinus cells in the spleen constitutes the greatest concentration of reticuloendothelial elements in the body. As such, the spleen has received considerable attention in cancer studies. Hypertrophy of the spleen in many types of tumor-bearing animals was noted shortly after Aschoff's delineation of the colloid-storing reticuloendothelial system. The atrophy of this system, frequently noted at autopsy on persons where death was due to malignancy, is also seen in animals bearing some types of tumors.

Investigations reported prior to 1942 have been reviewed by Stern and Willheim (*10*). Among the data that emerge from these reports are the following: a) The spleen is enlarged in most of the animals bearing tumors studied to date. b) The spleen is rarely the site of metastatic growth. c) Splenectomy or reticuloendothelial block variously affect growth of different tumors. d) Administration of preparations of spleen to tumor-bearing experimental animals and man has not consistently influenced growth of tumors. For a review of later evidence see the 1954 report of Chester Stock (*11*). e) Attempts to stimulate the spleen and reticuloendothelial system by physical, chemical, and biological methods have inconsistently affected tumor growth. f) Splenectomy and spleen therapy may variously affect the several spheres of body metabolism. These and related recent reports (*e.g.*, 12–14) indicate a possible relationship between

the spleen and cancer or some forms of cancer. The general function of the spleen has been discussed recently by Dameshek (*15*).

Begg (*16*) has noted that the enlarged spleen of tumor-bearing rats contains many large, clear round cells, megakaryocytes, and reticulum cells and that embryonal myelopoiesis is present. These changes in histologic composition may affect the chemical composition of the spleen.

Several investigations of the chemical composition of the spleen in tumor-bearing animals have been reported. Menten, Willms, and Wright (*17*) recorded an increase in the deoxyribonucleic acid-phosphorus content per splenic lymphocyte in leukemic mice. Busch (*18*) found that the ability of the spleen of rats to convert all administered acetate-1-C^{14} into nonvolatile (concluded to be nonacetate) radioactivity was not affected by tumor (including Walker carcinosarcoma 256) growth. LePage *et al.* (*19*) noted in Sprague-Dawley rats that starvation inhibited the enlargement of the spleen but not the growth of Flexner-Jobling carcinoma to any considerable extent. Stewart and Begg (*20*) force-fed diets containing 60 percent fat, carbohydrate, or protein to Sprague-Dawley rats bearing Walker carcinosarcoma 256 and found no change in the amount of enlargement of the spleen; their actual data suggest the possibility that force-feeding to a larger number of tumor-bearing rats might have revealed a statistically significant augmentation of splenic enlargement by high-protein diets.

On the basis of percent wet weight, citric acid concentration, which is high in various tumors, is found to be elevated in the spleen of rats bearing Walker carcinosarcoma 256 (*21*). Calculated as percent of wet weight, Aoki (*22*) recorded a mean decrease in total fatty acids, cholesterol, cholesterol esters, and phospholipide in the spleen of hepatoma-bearing rats. Evidence reviewed by Greenstein (*21*) indicates that the following enzymatic systems in the spleen are not affected by the presence of a tumor growing in the host: cytochrome oxidase, glyoxalase, deoxyribonucleodepolymerase, acid phosphatase, alkaline phosphatase, and total dehydrogenase.

The available evidence indicates that the spleen of animals bearing experimental tumors is usually enlarged, has active hematopoietic tissue, and may have an elevated citric acid concentration and possibly a lower lipide concentration than the normal spleen. Evidence obtained in the study reported above demonstrated a significant lowering of cholesterol concentration, no significant alteration in the mean level of other lipides, and an increase in water concentration per unit dry weight of spleen in albino rats bearing Walker carcinosarcoma 256.

Based upon mean changes in the lipide and water levels of carcass, testicle, and skeletal muscles of the hind limb in albino rats bearing Walker carcinosarcoma 256, Boyd *et al.* (*3*) suggested ". . . the production by the Walker tumor of a hydrolipotropic factor whose effect upon the tissues of the host is to make their water and lipid concentrations become more like those in the tumor . . ." A review of published values (*2, 7, 23*) indicates that the hydrolipotropic shift varies from organ to organ.

These previously published results are summarized in table 5. A majority of the values listed in table 5 followed the hydrolipotropic shift toward tumor levels. The shift was particularly evident in carcass, testicle, thymus, skeletal muscles of the hind limb, and duodenum. Bloor and Haven (*24*) obtained data which indicate that a similar shift occurs in the whole intestine ("from stomach to anus") of albino rats bearing Walker carcinosarcoma 256.

TABLE 5.—*An analysis of previously reported hydrolipotropic shifts in the organs of albino rats bearing Walker carcinosarcoma 256* (2, 7, 23)*

Tissue	Neutral fat	Total cholesterol	Free cholesterol	Phospholipide	Water
Carcass	(+) −80	(−) +54	(−) +56	(−) +25	(−) +45
Hind-limb muscles	(+) −65	(−) +84	(−) +121	(−) +13	(−) +30
Testicle	(○) 0	(−) +92	(−) +72	(○) 0	(+) −17
Brain	(○) 0	(+) 0	(+) 0	(+) 0	(−) 0
Heart	(○) 0	(−) 0	(−) 0	(○) 0	(−) +20
Lung	(○) 0	(○) 0	(○) 0	(○) 0	(−) +16
Skin	(+) −71	(−) 0	(−) 0	(−) 0	(−) 0
Duodenum	(+) −64	(○) 0	(○) 0	(○) +46	(−) +27
Liver	(○) 0	(−) 0	(−) +54	(+) 0	(−) +27
Thymus	(+) 0	(−) +141	(−) +77	(−) +47	(−) +32
Spleen	(−) 0	(−) −28	(○) −26	(−) 0	(−) +11

*The results are expressed as significant percentage changes in concentration, calculated as mean gm. per 100 gm. dry weight. Values in non-tumor-bearing litter mates are indicated in brackets as having been above (+) or below (−) or the same as (○) the tumor concentrations.

The concentrations of water and lipides in the spleen have been compared with those in Walker carcinosarcoma 256 (*7*), and a summary of the comparison is given in table 5. The increased mean concentration of water in the spleen was the only evidence of a shift toward tumor levels. The shift in mean cholesterol concentrations was actually away from the tumor level. The hydrolipotropic shift in the spleen was therefore unique among the organs studied to date. The results indicate a resistance by the spleen to the hydrolipotropic shift encountered in many other organs of the tumor-bearing host. Studies to date have been concerned with establishing the nature of these hydrolipotropic shifts. Their cause and significance remain to be investigated.

Summary

The spleens of 54 inbred albino rats bearing Walker carcinosarcoma 256 and of 54 non-tumor-bearing litter mates were analyzed for water and lipide composition. The weight of the spleen in tumor-bearing rats increased linearly with increasing log T/RC coefficients. The T/RC coefficient is the weight of the tumor divided by the weight of the residual carcass and the quotient multiplied by 100. The enlarged spleen of tumor-bearing rats contained significantly greater amounts of water, dry weight, total lipide, neutral fat, total fatty acids, total cholesterol, free cholesterol, and phospholipide. In terms of mean gm. per 100 gm. dry

weight, none of these constituents was altered except water concentration, which was significantly increased, and cholesterol (total and free) concentration, which was significantly decreased in the spleen of tumor-bearing rats. Cholesterol concentrations decreased linearly with increasing log T/RC coefficients. Water concentration was increased in animals bearing the smallest tumors studied. Neutral fat levels increased in animals bearing larger tumors. Sex of the host rat did not affect changes in mean levels but had some influence on correlations with tumor growth. The results demonstrated that a hydrolipotropic shift toward tumor levels, which occurs in tissues such as skeletal muscle and carcass of the tumor-bearing rat, is not evident in the spleen.

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Influence of Sex and Sex Hormones on Development of Hepatomas and Other Hepatic Lesions in Strain A × C Rats Ingesting 2-Diacetylaminofluorene[1,2]

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Bielschowsky (*1*) noted a much higher incidence of mammary tumors in female Wistar-strain rats than in males following the ingestion of 2-acetylaminofluorene (2-AAF). He concluded that the ". . . presence of the ovaries is a factor of great importance for the development of mammary cancer." In the same experiments, Bielschowsky observed more malignant hepatomas in male rats than in females (83% in 41 males compared with 31% in 36 females). Bielschowsky found the first hepatoma 21 weeks after beginning carcinogen administration. Stasney *et al.* (*2*) noted malignant lesions of the liver in 92 percent of Sherman-strain male rats and 55 percent of the females fed 2-AAF for 21 to 57 weeks. These authors noted, however, that this difference, apparent in the earlier weeks of the experiment, largely disappeared in the later weeks; *i.e.*, after 35 to 57 weeks of 2-AAF feeding 89 percent of the females and 100 percent of males had malignant lesions of the liver. The neoplastic changes were enhanced in males by administration of testosterone and chorionic gonadotrophin. There was indication that when estradiol, or possibly gonadotrophin, in pregnant mares' serum was administered to females, in addition to 2-AAF, these liver lesions appeared earlier than in control female rats. Kirby (*3*), studying 2-AAF carcinogenesis in Wistar-strain rats, noted that the female was less susceptible than the male to development of cirrhosis and liver tumors. Symeonidis (*4*) observed that females of M-520 and A × C strain rats had a higher incidence of liver tumors after ingestion of 2-AAF than females of the Buffalo, Sprague-Dawley, or Osborne-Mendel strains. He also found a higher incidence of liver tumors in A × C males (70.0%) compared with A × C females (only 46.4%). In studies of carcinogenesis in rats ingesting 2-AAF one should, therefore, recognize and give consideration to the characteristic susceptibility of the strain, as well as to the sex of the animals used. Leathem (*5*) reported that, in the Wistar-strain rats 6 months of age, 90 percent of the males and less than 10 percent of the females ingesting

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[2] Presented in part at a meeting of the American Association for Cancer Research, Cleveland, Ohio, April 27-29, 1951, and supported in part by grants from the Kansas Division of the American Cancer Society.
[3] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

2-AAF showed hepatic changes. This sex difference was not materially altered by equalizing the intake of food and carcinogen through paired feeding.

Rumsfield, Miller, and Baumann (*6*) have found that the ingestion of 3′-methyl-4-dimethylaminoazobenzene and 4′-fluoro-4-dimethylaminoazobenzene induced more cirrhosis and liver tumors in male rats than in females, whereas 0.06 percent 4-dimethylaminoazobenzene in the diet was equally potent as a carcinogen for the liver in both sexes. In *in vitro* studies, where slices or homogenates from livers of male or female animals were used, they found no sex difference in the ability to cleave these carcinogens, except late in the experiment when the male rats receiving 3′-methyl-4-dimethylaminoazobenzene showed less ability to detoxify the dyes.

A high incidence of liver tumors has been reported by Morris *et al.* (*7*) in Minnesota-strain rats ingesting 2-diacetylaminofluorene (2-diAAF). There was no evidence in this report to indicate a greater susceptibility to liver-tumor induction in one sex over the other. In subsequent, unpublished studies (*8*) on early induction of liver tumors with 2-diAAF in this laboratory, a much higher incidence of such tumors was noted in male than in female rats. This observation led us to make a more comprehensive study of the influence of sex and sex hormones on the development of hepatoma in the rat (*9*).

Procedure

Inbred A × C rats of both sexes were used in a series of three experiments. The rats varied from 2 to 4 months of age when the experiments were started. Six different groups were studied: intact males, intact females, castrated males, castrated females, castrated males receiving diethylstilbestrol, and castrated females receiving testosterone propionate. The pellets of estrogenic material used in the castrated male rats consisted of a 1:3 mixture of diethylstilbestrol and cholesterol. The pellets used in the castrated female rats consisted of a 1:3 mixture of testosterone propionate and cholesterol. The pellets for both groups were administered subcutaneously in a dose of 20 mg. per 100 gm. of body weight, representing at the time of administration a potential dose of 5 mg. of hormone per 100 gm. of body weight. The pellets were administered 10 days after castration on the first day of the experiment. Approximately 0.09 mg. of diethylstilbestrol and 0.13 mg. of testosterone propionate were absorbed per rat each week. Each group in the first experiment received the basal diet no. 165 (see table 1) that contained 0.05 percent of 2-diAAF continuously throughout the experiment. Each group in the second experiment received the same carcinogen-containing diet for 4 weeks, followed by a 3-week rest period on diet no. 214 (see table 1). In a second period of 3½ weeks the carcinogenic diet was again given, and this was followed by feeding of diet no. 214 without carcinogen for the duration of the experiment. In a third experiment, castrated females, with and without supplementary testosterone, were fed as follows:

2 weeks on carcinogenic diet no. 165, 2 weeks on diet no. 214, and then alternately, at weekly intervals, on the carcinogenic and basal carcinogen-free diet for the duration of their lives.

The second and third experiments were designed to increase the survival time of the animals. Periodic observations were made throughout experiments II and III from biopsies taken during laparotomy at intervals beginning 6 weeks after the start of the experiments. Laparotomy was carried out under ether anesthesia. At the termination of the experiment or at the time of death of the animals, an autopsy was also performed unless extensive autolysis had occurred. In the latter case, the animals

TABLE 1.—*Composition of diets*

Constituents	Amount	
	Diet no. 165	Diet no. 214
	(*gm.*)	(*gm.*)
Vitamin-free casein*	1, 200	1, 800
Cerelose † (+ 2-diAAF)	7, 470	5, 880
Salt mixture 185a (*10*)	300	300
Lard ‡	—	2, 000
Coconut oil (+ vit. A + E) §	25	—
Glycerol	1, 000	—
Linoleic acid	5	—
Choline	—	20
Vitamins ‖	(*mg.*)	(*mg.*)
Thiamine · HCl	30	30
Riboflavine	20	20
Pyridoxine · HCl	20	20
Calcium pantothenate	140	140
Niacin	—	200

* From Labco Division, Casein Co. of America, 340 Madison Ave., New York, N. Y.

† Five gm. of 2-diacetylaminofluorene were dissolved in acetone and mixed with 1 kg. of the cerelose. The solvent was allowed to evaporate, and the proper amount of the mixture was incorporated into the diet.

‡ Contained 50,000 units vitamin A ester and 500 mg. α-tocopherol acetate.

§ Contained 30,000 units vitamin A ester (kindly furnished by Distillation Products, Inc., Rochester, N. Y.) and 1.0 gm. α-tocopherol acetate.

‖ All the vitamin constituents listed were thoroughly mixed separately with cerelose and an aliquot added to the diet mixture.

were excluded from the experiment unless adequate biopsy data were available for evaluation of the state of the liver.

The lesions in the liver, whether obtained by biopsy or at autopsy, were observed and described grossly as they developed, and tissues were fixed in 10 percent formalin or Helly's fluid, embedded in paraffin, sectioned, and stained routinely with hematoxylin and eosin.

Experimental Results

The experimental results and intake of 2-diAAF are summarized in table 2 and text-figure 1. In table 2 the hyperplastic and neoplastic lesions are tabulated according to the most advanced lesion found in the liver, and those animals with small hepatomas or carcinoma are not included in the figures for hyperplastic nodules, although their livers almost always contained such nodules as well.

TABLE 2.—*Influence of different feeding regimens, sex, and sex hormones on induction of liver lesions by 2-diAAF*

Experiment	Sex	Weight change (*gm.*)	Intake 2-diAAF (*mg./kg. body wt./wk.*)	Cirrhosis	Hyperplastic nodules*	Small hepatoma
I.—Continuous feeding of 2-diAAF	Males					
	Intact	−21	166	5/5	2/5	3/5
	Castrate	−15	164	3/6	0/6	2/6
	Castrate + stilbestrol	−34	212	1/7	2/7	2/7
	Females					
	Intact	−12	213	1/8	0/8	1/8
	Castrate	−12	214	1/8	0/8	0/8
	Castrate + testosterone	−17	217†	7/7	0/7	6/7
II.—Interrupted feeding of 2-diAAF	Males					
	Intact	−33	123‡	8/8	0/8	7/8
	Castrate	+12	129	2/7	1/7	1/7
	Castrate + stilbestrol	+5§	170	1/5	1/5	0/5
	Females					
	Intact	+30	159	1/6	0/6	0/6
	Castrate	+31	165	1/12	0/12	0/12
	Castrate + testosterone	−10	135	3/4	0/4	3/4
III.—Alternate feeding of 2-diAAF	Females					
	Intact	+5	126‡	7/8	7/8	1/8
	Castrate + testosterone‖	+24	126	8/10	4/10	2/10

*Not tabulated in cases in which small hepatomas or carcinomas were present.

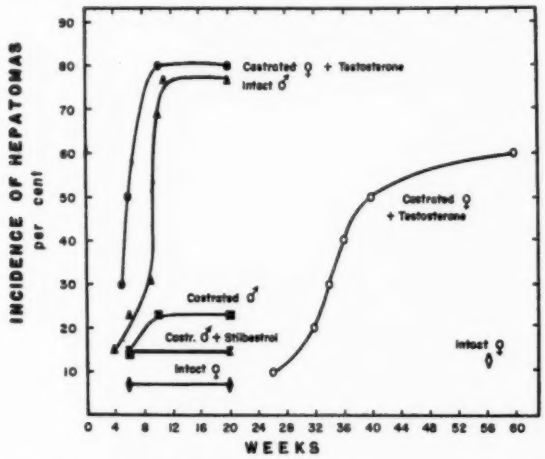
†Intake figures for the last week not included in calculation or average because animals were dying off rapidly and ate abnormally little during that week.

‡Amounts consumed only during the weeks while carcinogen was being fed; gives no indication of food intake during weeks on noncarcinogenic diet.

§Despite the final net gain these animals consumed more food and carcinogen, yet they lost considerably more weight during the early part of the experiment, than any from other groups.

‖Hepatocellular carcinoma was found in 4 of 10 female castrates given testosterone in group III. In one animal the tumor also contained both neoplastic areas resembling bile ducts and hepatic parenchymal cells; it was classified as a malignant cholangiohepatoma.

It was evident in the course of all three experiments that rapid loss in weight occurred during the period of 2-diAAF ingestion. During the periods on the noncarcinogenic diet in experiments II and III, the rats usually regained almost all the weight lost during ingestion of the carcinogen, except that the group of castrated males given stilbestrol in experiment II failed to gain as well. In experiment III, there was an initial average loss in weight of 12 gm. per animal for each of the two groups, but, after the first few weeks of the experiment, both the intact females and the castrated females given testosterone propionate slowly regained their initial weight and maintained it under the alternate feeding regimen. The castrated females given testosterone, as a group, actually exceeded their average original weight by 24 gm. per rat at the end of the experiment.



TEXT-FIGURE 1.—Crude rates of development of hepatomas in rats following the ingestion of 2-diacetylaminofluorene. The solid points represent findings in experiments I and II; the open points represent findings in experiment III.

The lesions that developed in the livers of these animals in each of the three experiments generally can be classed in two categories—those with changes principally involving the biliary ducts and those with changes mainly involving the hepatic parenchymal cells and lobular structure. Actually, in almost all instances changes in the biliary tree and hepatic parenchyma developed concurrently, and this separation is made solely for the purpose of description and is in no way meant to detract from the importance of their interrelationships.

The earliest changes in the biliary tree consisted of the proliferation of small bile ducts as thin strands of spindle-shaped cells in a double row. These bile ducts had oval nuclei arranged in tandem that extended from portal areas into the adjacent parenchyma (figs. 1 and 2). Generally, the lumen was either collapsed or as yet unformed. This lesion, as it became more advanced, was accompanied by some portal fibrosis with accentua-

tion and also, at times, some distortion of the lobular architecture—forming the picture of cirrhosis (fig. 3). Associated with this lesion of cirrhosis, macrophages containing brown ceroid pigment were seen in the portal areas, and some of the larger bile ducts became obstructed, distended, and cystic. As dilation increased, the lining of the cysts became flattened to such an extent that the cells resembled endothelial cells. In some instances, it was apparent by studying changes present in the same liver in somewhat different stages of development that the cystic spaces arose from obstructed, smaller bile ducts which had grown into the hepatic parenchyma. In other instances, larger bile ducts with columnar-lining epithelium were involved. There were a few loculated collections of cysts that seemed to involve both sizes of ducts (fig. 4). The smaller ducts, when the cysts were well formed, often trapped groups of hepatic parenchymal cells in their midst.

Apart from these areas, cystic areas of another type, rarely seen, were found which consisted of dilated sinusoidal spaces within the hepatic parenchyma that were lined by sparse endothelial cells, at times difficult to make out, and it almost seemed that hepatic parenchymal cells formed the lining. These spaces were not filled by red blood cells, as one would expect if they were connected with the vascular sinusoids, but instead they contained only precipitated protein. These curious telangiectatic lesions were interpreted as nonproliferative lesions secondary to obstruction and therefore are not included in the subsequent analysis.

Changes in the hepatic parenchyma were characterized by a minimum of cytologic degeneration (fig. 5). A few foci of fatty change were sometimes evident and in rare instances there were foci of acidophilic necrosis. However, the latter were so uncommon that it could be assumed that they were unrelated to the diet or carcinogen and probably represented coincidental infection. Despite the almost complete lack of evident necrosis, there was apparent shrinkage of liver cells and nuclei in the central areas and proliferation of cells in the periportal areas (figs. 6 and 7). These proliferating cells were conspicuous because of increased amounts of basophilic material in their cytoplasm. These changes progressed with the development of cirrhosis, and the foci of periportal hyperplasia developed into larger areas of hyperplasia that occupied most of a lobule and sometimes formed nodules which compressed and distorted the adjacent hepatic parenchyma (figs. 8–12). In some of these nodules the trabeculae were formed by double columns of parenchymal cells, such as those described in regeneration in the study of the histogenesis of cirrhosis by Popper and Elias (*11*) and in hepatocarcinoma by Elias (*12*).

The more well-developed nodules began to lose all semblance of cordlike structure; their cells showed variation in size, shape, and staining; in the central areas there appeared to be loss of stroma and cohesiveness, and occasional degenerating and necrotic cells could be seen. These changes suggested neoplastic tumor formation and transition to small hepatomas (figs. 13 and 14). In the early stages of hyperplasia, mitotic figures were uncommon but binucleate cells were frequent. Later, mitotic figures

were frequent in many of the larger areas, and the nodules of hyperplasia and some of the mitotic figures showed lagging of chromosomes and similar abnormalities. Finally, large malignant hepatomas of parenchymal cells developed—some had a pseudoacinar pattern (fig. 15), others had a solid or trabecular pattern (fig. 16). In one case, there were ductlike structures resembling cholangiocarcinoma comprising part of the tumor and it was regarded as a malignant cholangiohepatoma.

For convenience in analyzing the results, each of the two categories has been broken into somewhat artificial classifications according to the stage of development of the lesions at the time of examination. Changes in the biliary tree were grouped under (1) slight bile-duct proliferation or (2) cirrhosis and cyst formation (no tumors of purely bile-duct origin were encountered in this small series). Changes in hepatic parenchymal cells were classified under (1) foci or areas of hyperplasia, (2) nodules of hyperplasia, (3) nodules with loss of lobular architecture, compression of adjacent parenchyma, and atypicality of cells—probably early tumors—and referred to in the text as small hepatomas, and (4) distinct malignant hepatomas of liver-cell type (hepatocellular carcinoma) or, in one instance, a malignant cholangiohepatoma.

Experiment I

In this experiment, in which the animals were continuously fed the carcinogens (table 2), the groups which developed lesions did not fare well and began to die at the fifth week. The experiment was therefore terminated at the sixth week. Already there was a profound difference in the changes in the livers of the intact male and female animals. Three out of 5 males had multiple small hepatomas of liver-cell type in 6 weeks; in addition all 5 animals had extensive, small bile-duct proliferation and cirrhosis (fig. 2). The 2 animals without hepatomas did show some nodular hyperplasia and slight atypicality of cells in a few areas. During the same period, 7 out of 8 of the intact females had essentially normal livers with no evidence of cirrhosis or hyperplasia; the eighth animal had 2 small hepatomas and extensive portal cirrhosis entirely similar to that in the males.

This difference in tumor incidence according to sex existed despite a higher intake of 2-diacetylaminofluorene per animal among the females. Each of the males consumed an average of 166 mg. of 2-diacetylaminofluorene per kg. of body weight per week, or an average total intake per rat of 108 mg. for the total period. By comparison, the average female consumed 213 mg. per kg. of body weight, or a total average intake of 116 mg. per animal for the same period. As might be expected, the lower intake of food by the males was reflected in a slightly greater loss of weight amounting to an average of 21 gm. per rat, while the females lost only 12 gm. of weight per animal during the 6-week experimental period.

Interestingly, this difference in the sexes was not completely abolished by castration a week or two before beginning the feeding of the carcinogen. Two of 6 castrated males developed small hepatomas with cirrhosis, a

third had cirrhosis but no tumors, and the remaining 3 had essentially normal livers, although 2 of them showed some slight bile-duct proliferation. The effectiveness of the castration was evident, both anatomically and functionally, in these animals by the shrunken and nonfunctional appearance of the prostate and seminal vesicles. The livers in the 8 castrated females, by contrast, were essentially normal, except for 1 animal with cirrhosis and 2 others with only slight bile-duct proliferation. None of these animals showed areas of hyperplasia or indication of hepatic parenchymal proliferation.

A review of their carcinogen consumption reveals essentially the same intake by the castrated animals as the intact animals of the same original sex. The intake of castrated males still did not approach that of the females. As might be expected there was a slightly greater average weight loss in the male than in the female castrates.

When the male castrates were given supplementary estrogen by pellet simultaneously with the beginning of the carcinogenic diet, the pattern of liver changes was essentially unchanged from that of the male castrates without estrogen. Two of the 7 rats developed small hepatomas and 1 of these had a cirrhotic liver; 2 rats had hyperplastic nodules (fig. 9) and the remaining 3 were essentially normal, with only slight bile-duct proliferation and hyperplastic areas in 2. However, this group consumed more carcinogen per kilogram of body weight than either the intact or castrated male animals without estrogen and equaled the females in intake. Paradoxically, they lost more weight (average 34 gm. per animal) than any of the other groups for the 6-week period, which suggests some antianabolic or toxic effect of the stilbestrol.

When female castrates were given testosterone pellets to simulate the male endocrine situation, they still behaved essentially like castrated females with respect to carcinogen consumption and weight loss. However, the pathologic changes in the liver were profound in this group and were similar to those in the group of intact males. All the animals had cirrhosis, and 6 of the 7 had what were interpreted morphologically as small hepatomas.

Experiment II

Somewhat larger animals, ranging in average initial weight from 127 to 169 gm., were used for the second experiment (table 2). During the 3½- to 4-week periods on the carcinogen their average weekly consumption of carcinogen was less than for the smaller animals (101 to 118 gm.) of experiment I. Also, the loss of weight of these larger animals was greater, but all groups, except the castrated males given stilbestrol, regained their lost weight during the periods on the better diet without carcinogen. However, due to the longer period of carcinogen administration, the total intake was slightly greater than in the first experiment.

The pathologic changes in this experiment, as might be expected, were slightly slower in developing but showed the same correlation with sex and the presence of androgen as in the previous experiment.

In the group of 8 intact males all had normal livers when biopsied between the fifth and sixth weeks, but by 10 and 11 weeks 7 of the 8 had cirrhosis and small hepatomas (figs. 13 and 14). The remaining animal had cirrhosis at the time of biopsy, 14 weeks after starting the carcinogen, and was re-examined with laparotomies and biopsies at 5 months and 1 year. Although this animal continued to have cirrhosis, it failed to develop nodules of hyperplasia or a hepatoma.

In the group of 6 intact females, 3 were biopsied at 6 weeks and were normal; all 6 were examined at 16 weeks after the first exposure to carcinogen and 4 were normal. The other 2 died; in 1 there was a focus of hyperplasia of parenchymal cells; in the other, not previously biopsied, there was an abscess in the liver with fibrosis in the area of the abscess and some portal cirrhosis elsewhere with moderate, small bile-duct proliferation. The 4 surviving animals were sacrificed at 5 months after the start of the experiment and had essentially normal livers, except for an area of hyperplasia in the liver of 1 animal. In none of these animals did the hyperplastic changes form distinct nodules with distortion of surrounding liver parenchyma.

In the group of 7 castrated males, repeated biopsies revealed normal livers for a period of 5 months in 3 animals. Only 1 animal had advanced portal cirrhosis and multiple small hepatomas, and this animal died 11 weeks after the start of the experiment. Two animals had cirrhosis when biopsied at 3½ months, which in 1 animal had largely disappeared at 5 months, but then there were areas of hyperplasia. The other animal also had, in addition to cirrhosis, nodules of hyperplasia at 5 months. The remaining animal had a biopsy which was normal at 3½ months, but there were focal areas of hyperplasia in the parenchyma of the liver when the animal died 2 weeks later.

In 12 castrated female rats repeated biopsies failed to reveal any notable abnormalities in the livers, except for small non-nodular areas of hyperplasia which were found at autopsy in 2 animals when all the animals were sacrificed 5 months after beginning the administration of carcinogen. One of these 2 animals also had portal cirrhosis.

In 5 castrated male rats given stilbestrol, 1 animal had a nodule of hyperplasia in the liver 5 months after the start of the experiment which was not evident at the time of biopsy 6 weeks previously (fig. 12). Two other rats had tiny foci of periportal hyperplasia, also first detected at autopsy when the experiment was terminated 5 months after its start. The remaining 2 rats had normal livers throughout the experiment.

In the group of 9 castrated females given testosterone, all were examined and biopsied 6 weeks after the start of the experiment and the liver was normal in 7. Two of the rats had cirrhosis and hyperplastic nodules. Unfortunately, 5 of the 9 rats, inadvertently, were not returned to the carcinogenic diet at the scheduled time and therefore had to be excluded from the experiment. Three of the 4 rats, returned to the diet as planned, had both cirrhosis and small hepatomas when they died at 10 weeks. The fourth rat was re-examined and biopsied at the 14th week and

sacrificed at the 20th week, but the liver revealed only a few small foci of hyperplasia.

Experiment III

It was apparent that the intact females were rather highly resistant to the carcinogen compared with the intact males or castrated females given testosterone. There was some question as to whether this represented permanent resistance or merely a slowing of the process of carcinogenesis. Consequently, it was decided to carry out a limited third experiment using 8 intact females, alternating weekly periods of administration of carcinogen, in the low protein-low choline diet with periods of administration of a higher protein-higher choline diet without carcinogen. In this manner, the health of the animals could be maintained for a much longer time. For comparison, 10 females were castrated, pellets of testosterone propionate were implanted subcutaneously, and the animals were placed on a similar regimen. A summary of the results is given in table 2.

Laparotomies with biopsies of the liver in the 8 intact females, 8 weeks after starting the carcinogenic diet, revealed normal livers in all the rats. Similar studies in the 10 castrated females given testosterone revealed that 6 of the animals already had portal cirrhosis and 4 had hyperplastic nodules. After 6 months all the intact females had hyperplastic nodules (fig. 10), with portal cirrhosis in 5 of the 8 animals. At the same time, the findings in the group of 9 surviving castrated females given testosterone were entirely similar, except that all the animals had cirrhosis and 1 rat died with a small hepatoma. At 7½ months another rat in this group died with a large, poorly differentiated hepatoma which was successfully transplanted subcutaneously to rats of the same strain (fig. 16). This tumor has since been transplanted for over 66 generations. By 8½ months, 2 more of the castrated testosterone-treated female rats had similar large hepatomas (fig. 15), 1 of which has been successfully transplanted subcutaneously for over 40 generations. In the same period, however, no change had occurred in the status of the intact females. Within the following 1½ months 2 more castrated testosterone-treated females developed hepatomas, 1 of which had peritoneal implants of tumor and metastases in several abdominal lymph nodes and in the lungs. The latter tumor was also transplanted serially for several generations.

At the end of the experiment, 13½ months after the start, a total of 6 of 10 castrated females given testosterone developed hepatomas, 5 of which ranged from 1 to 4½ cm. in diameter at the widest part. In the only three instances in which transplantation of the tumors was attempted it was successful. The tumors in this group were the largest and least differentiated morphologically, and the only ones in the three experiments in which there was biological evidence of their neoplastic nature. Unfortunately, no transplantation was attempted with any of the small, presumably early hepatomas in this or the previous experiments. One large hyperplastic nodule lacking cytologic atypicality was transplanted subcutaneously and intramuscularly and failed to grow.

Although 7 of the 8 intact females developed cirrhosis, in contrast, only 1 of the 8 had a small hepatoma, and this was hardly comparable to those in the castrated females treated with testosterone. It was noteworthy that all of these animals did have hyperplastic nodules, which suggests the possibility that tumors might have developed later had these animals survived longer. The great difference in susceptibility of the two groups was not related to carcinogen intake since it was similar in both groups on a per-rat basis, and it was actually slightly greater on the per-kilogram-of-body-weight basis in the intact females during the later weeks of the experiment.

Discussion

An attempt was made in a series of three experiments to determine the role of sex and sex hormones on the incidence of hepatomas in A × C rats fed 2-diacetylaminofluorene with a low-fat, low-protein, low-choline, high-carbohydrate diet. The data in table 2 summarize the average intake of the carcinogen for the different groups during the several experiments outlined above. Since there was variation in the size of the animals used and since the experimental conditions in experiments II and III also varied, the 2-diAAF intake has been expressed as average weekly intake in mg. per kg. of body weight. The 2-diAAF intake, as shown in table 2, indicated much the same intake of carcinogen for the various groups in experiments I and II, and, therefore, the intake did not appear to be the limiting factor which affected the induction of hepatomas in these experiments. The average intake of 2-diAAF per week per kg. of body weight ranged from 120 to 217 mg., with the most susceptible groups generally having lower intake values; these were reflected in greater weight loss than in the other groups with the possible exception of the castrated males given stilbestrol. Of course, these figures refer to the weeks during which the animals were given the carcinogenic diet. The actual average total intake per rat was slightly greater in experiment II with the rest period than in experiment I with continuous feeding, because the total time on the carcinogenic diet was slightly longer.

The average intake of the two groups in experiment III was identical during the alternate weeks when carcinogen was ingested, averaging 126 mg. per kg. per week, which is similar to the intake of males, both intact and castrated, and of castrated females given testosterone in experiment II. The lessened toxicity of this regimen was reflected in the ultimate weight gain of these two groups in experiment III, which was terminally somewhat more in the castrated females given testosterone—probably due to the development of the liver tumors. The total intake per rat of 2-diAAF in experiment III with alternate feeding was 2 to 4 times that of rats in the continuous- or interrupted-feeding experiments I and II, yet the percentage of animals developing tumors was slightly less than in the previous experiments.

Although the rate of evolution of the lesions in the liver varied with the mode of administering the carcinogen, being most rapid with continuous

feeding and slowest with alternate feeding, the types of lesions developing in each group in all three experiments were essentially the same. The rate of development of the tumors in experiments I and II, although somewhat slower in experiment II, was reasonably comparable and the number of animals in experiment I was small, so that text-figure 1 represents composite curves of tumor development in both experiments I and II. The curve in text-figure 1 representing the incidence of tumors in castrated females given testosterone with alternate feedings in experiment III shows that the rate of development of tumors was very much slower, and the height of the peak is not quite so great as that obtained in experiments I and II for the corresponding animals.

The intact males and castrated females given testosterone developed cirrhosis, cystic areas, areas and nodules of hyperplasia, and small hepatomas in a high percentage of instances. This was in sharp contrast to the intact and castrated females and castrated males, both with and without supplementary diethylstilbestrol, in which relatively few morphologic changes were found in the liver.

Castrated males retained some of the susceptibility to carcinogen of intact males but developed the lesions in a much lower incidence. Supplementary estrogen appeared to have little protective effect but merely an antianabolic effect on body weight. Castrated females given testosterone more closely resembled intact males in the development of lesions, although they developed somewhat more slowly. In experiments I and II the incidence of such tumors reached that in the intact males, but not quite as high an incidence was attained in experiment III; however, no intact males were subjected to this regimen for comparison.

The lesions in all instances in experiment I were diagnosed as small hepatomas purely on a morphologic basis but, on this basis, it is difficult to know with certainty when a lesion becomes irreversibly neoplastic biologically. All the animals died or were sacrificed before it was possible to be certain biologically that one was dealing with malignant hepatomas. Experiments II and III were therefore set up, using intervals on an adequate diet with alternate feeding of the carcinogenic diet. In both experiments II and III, lesions developed somewhat more slowly and the animals showed less weight loss and survived much longer. In an attempt to correlate morphologic findings with biological behavior of the tumors, laparotomies were performed on a number of the animals in these experiments and serial biopsies, particularly of gross lesions, were studied.

The findings by study of serial biopsy lend further weight to the significance of the demonstrated role of maleness and male sex hormone in the carcinogenesis of tumors of the rat liver with 2-diacetylaminofluorene by revealing the earlier development of hepatic lesions as well as their higher incidence in these groups. On the other hand, it would seem apparent that the presence of estrogen does not exert any definite protective action against carcinogenesis.

It was hoped that the prolonged survival time in experiment III would reveal whether or not the hormonal influence was merely an accelerant in

the process of hepatic carcinogenesis. The results of this experiment failed to yield a conclusive answer to this question and one can only say that, after 10 months under the conditions of this experiment, there was still a striking difference in incidence of hepatic tumors in castrated females given androgen from those without supplementary hormone; and by prolonging the survival period to this degree, despite the increased total dose of carcinogen, no increased incidence was demonstrated.

This study takes on added significance with the subsequent finding by Griffin and others (*13*) of the importance of hypophysectomy in preventing or delaying the development of hepatomas in rats given dimethylaminoazobenzene. Griffin and his co-workers (*14*) have recently stated that preliminary results indicate that hypophysectomy also prevents the induction of liver and ear-duct tumors in rats given 2-diAAF; however, difficulties arising from low food intake and 2-diAAF toxicity necessitate further studies. We are just completing some experiments which also bear out this protective effect of hypophysectomy.

The important role of the adrenocortical hormones must also be evaluated. Fortunately, Symeonidis and his co-workers (*15*) last year published some work, which was carried out simultaneously with this study, in which deoxycorticosterone had a sparing action on the development of tumors of the liver in rats given *p*-dimethylaminoazobenzene. Possibly this exerts its effect by inhibiting the pituitary production of adrenocorticotrophin or conceivably gonadotrophin. The work of Robertson and his colleagues (*16*) would suggest that adrenocorticotrophic hormone is the most important of the pituitary hormones for hepatic carcinogenesis, but the number of animals reported prohibits final interpretation, and further study is required.

It is evident from review of the incidence of primary tumors of the liver in man that the importance of maleness and testosterone may not be limited to rats. In man, the difference in incidence of primary carcinoma of the liver between the sexes is well known and the predominance in males varies from 2 : 1 in children (*17*) to 6 : 1 in adults (*18*). Here, too, however, the role of the adrenal cortex, thyroid, and pituitary remain undetermined.

Summary and Conclusions

The role of sex and sex hormones in A × C rats in the induction of cirrhosis and hepatomas by ingestion of 2-diacetylaminofluorene (2-diAAF) has been studied in intact and castrated rats of both sexes, in castrated males given diethylstilbestrol, and in castrated females given testosterone propionate. Morphologic lesions interpreted as small, early hepatomas of the liver developed within 5 to 6 weeks after continuous ingestion of a diet containing 0.05 percent 2-diAAF. Most of the lesions were confined to the livers of intact males and castrated females treated with testosterone propionate. Intact females, castrated males, castrated females, and castrated males treated with diethylstilbestrol were more resistant.

Continuous ingestion of 2-diAAF for 4 weeks, followed by regular or intermittent rest periods on the control diet of 1 or more weeks' duration,

increased survival time and delayed the appearance of hepatomas in the susceptible animals. In one experiment, a somewhat lower incidence of tumors was obtained in castrated females given testosterone over that obtained in similar animals given higher doses of carcinogen early in the course of the administration.

The development of lesions was followed by means of repeated laparotomy and biopsies in experiments in which the carcinogen was fed intermittently. Lesions short of hepatomas were characterized histologically by proliferation of small bile ducts and portal cirrhosis, associated with areas of hepatic-cell hyperplasia, often without distinct lobular architecture. In those few animals in which a diagnosis of small hepatomas was made by biopsy, there were always similar or more advanced neoplastic lesions in the liver at autopsy. Although the validity of the microscopic criteria for early hepatoma seemed to be borne out by the subsequent course of the lesions, the number of instances in which serial biopsy material was available for analysis was insufficient. Unfortunately, no attempt was made to transplant any of the small, gross lesions which were microscopically interpreted as early hepatomas. Successful subcutaneous transplants of three of the more advanced lesions were made serially to other rats of the same strain.

From each of the experiments it is evident that, at least under the conditions of these experiments, the presence of androgen, like the role of estrogen in breast tumors of mice, is important for the development of cirrhosis and hepatomas in the rat. There is no evidence that estrogen has additional protective action against the development of either cirrhosis or hepatomas in castrated male rats.

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PLATE 78

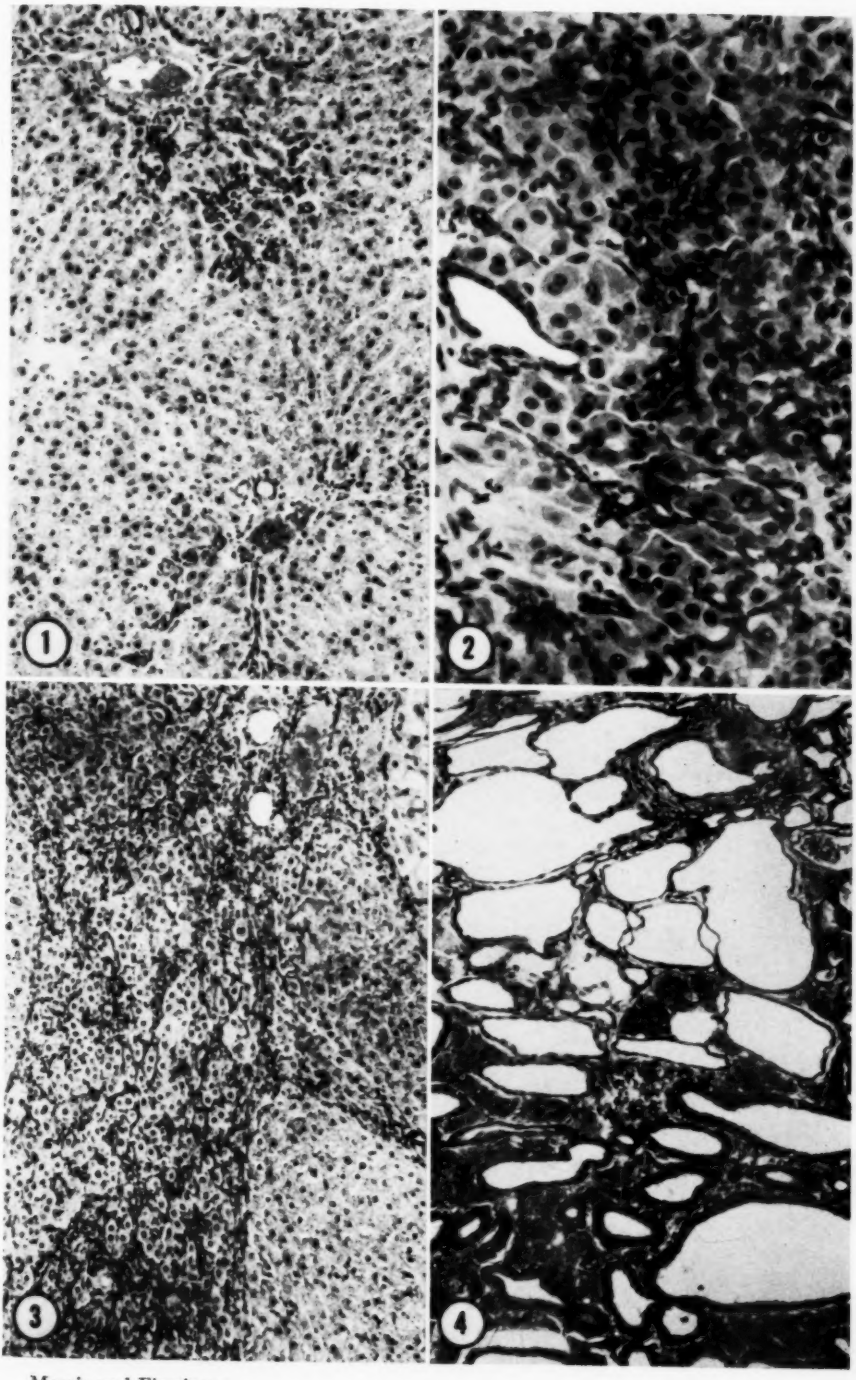
All of the photomicrographs are of sections stained with hematoxylin and eosin.

FIGURE 1.—Slight bile-duct proliferation in a castrated male rat in experiment II, 5 months after beginning 2-diAAF administration. This represents the first notable change in the bile ducts and precedes the development of cirrhosis and cyst formation. × 145

FIGURE 2.—Increased bile-duct proliferation with beginning of isolation of liver parenchymal cells in a castrated male rat in experiment I, 6 weeks after beginning 2-diAAF administration. Lumina of proliferating ducts generally are poorly formed; however, one small duct is somewhat dilated. × 335

FIGURE 3.—Cirrhosis of the liver in an intact male rat in experiment II, 14 weeks after beginning 2-diAAF administration. Parts of lobules are shown separated from one another by proliferating, poorly formed, small bile ducts and capillaries, with only a slight increase in reticulum and fibrous tissue compared with similar lesions in man. × 105

FIGURE 4.—Bile-duct proliferation with cyst formation developing in a castrated female rat given testosterone in experiment III, 13½ months after beginning 2-diAAF administration. Area reveals some dilated ducts lined by cuboidal or low-columnar epithelium and others, more dilated, lined by flattened cells simulating endothelium. × 105



Morris and Firminger

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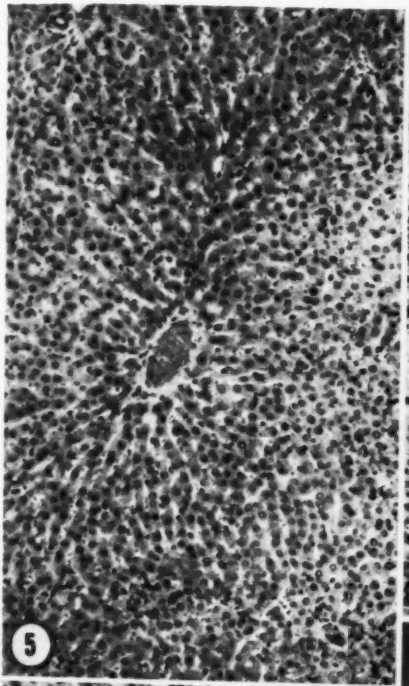
PLATE 79

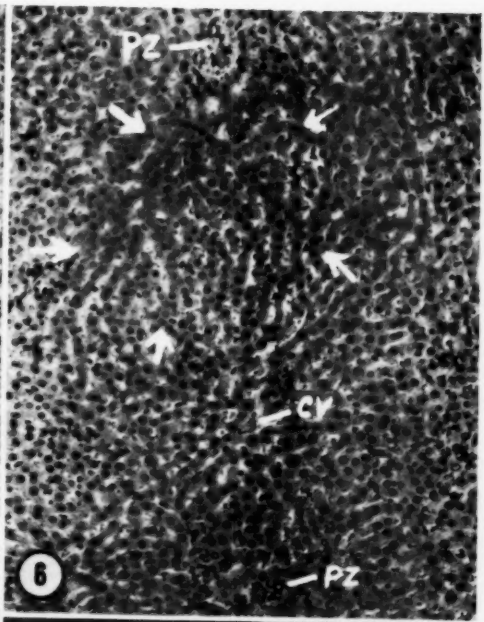
FIGURE 5.—Normal area of liver in an intact female rat in experiment III, 6½ months after beginning 2-diAAF administration. No distinct proliferation of either parenchymal cells or bile ducts is present in this area. × 135

FIGURE 6.—Another area in the liver from the intact female rat in experiment III shown in figure 5. There is a poorly demarcated focal area in the periportal region (indicated by *arrows*) characterized by an increased number of small cells with more deeply stained cytoplasm. This area is interpreted as a focus of hyperplasia. CV indicates central vein and PZ refers to portal zones. × 135

FIGURE 7.—Periportal hyperplasia of parenchymal cells developing in a castrated female rat given testosterone in experiment III, 6 months after beginning 2-diAAF administration. The hyperplastic cells are enlarged and contain an increased amount of cytoplasmic basophilic material. × 135

FIGURE 8.—Photograph illustrating gross difference in the liver of a male A × C rat (*A*) from that of a female rat similarly treated (*B*). Both rats were fed the carcinogenic diet for 5 weeks and then alternately the carcinogenic and noncarcinogenic diets for 8 weeks, so that 2-diAAF was ingested for a total of 9 weeks. Note the many discrete white nodules in all lobes of the liver of the male rat and their complete absence in the liver of the female.




PZ
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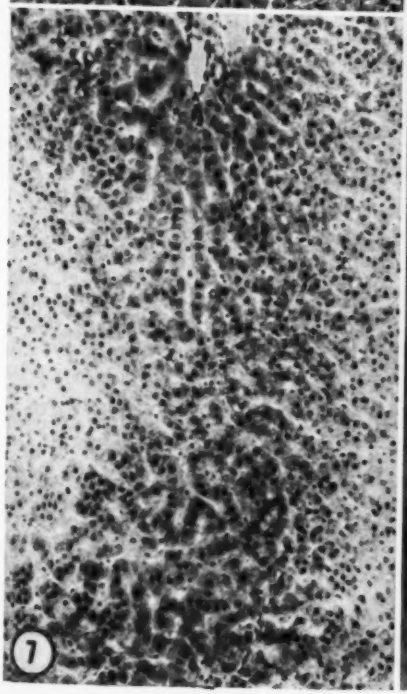


A

PLATE 80

FIGURE 9.—A small hyperplastic nodule in the liver of a castrated male rat given stilbestrol in experiment I, 6 weeks after beginning 2-diAAF administration. This nodule is composed of a rather disorderly array of cords of well-differentiated liver cells which in this instance have a rather vacuolated cytoplasm. Note the distortion of the lobule and compression of the surrounding liver cords. CV indicates the central vein of the lobule and PZ, the region of the portal zones. × 155

FIGURE 10.—Well-defined trabecular formation with Elias' double columns (*12*). The trabeculae in some areas connect with the adjacent liver cords, yet there is distinct distortion of some of the adjacent parenchyma by the nodule. This hyperplastic nodule was found in an intact female rat in experiment III, 9 months after the start of administration of 2-diAAF. × 140

FIGURE 11.—A hyperplastic nodule in the liver of a castrated female rat given testosterone in experiment III, 6 months after beginning 2-diAAF administration. The nodule is composed of fairly small cells with more dense, basophilic cytoplasm. The trabecular pattern, although somewhat obscure, can still be made out and the compression of the adjacent liver parenchyma can be seen. × 135

FIGURE 12.—Hyperplastic nodule formed in the liver of a castrated male given stilbestrol in experiment II, 5 months after beginning 2-diAAF administration. The trabecular formation is obscure, but there is more variability in cytologic characteristics and even some atypicality; however, these changes seemed somewhat less marked than those seen in the small hepatomas. × 155

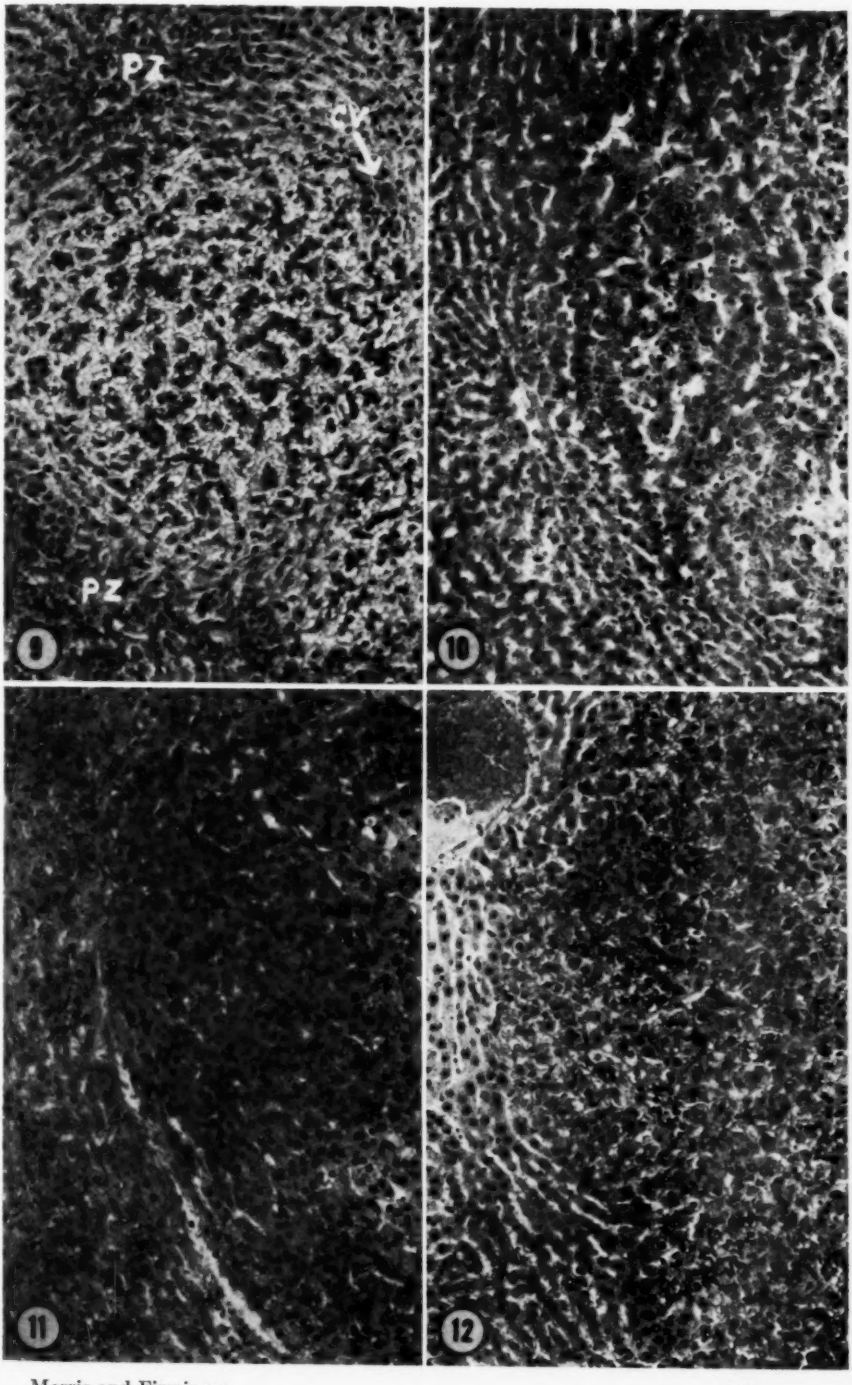

PZ
CK
PZ
9
10
11
12

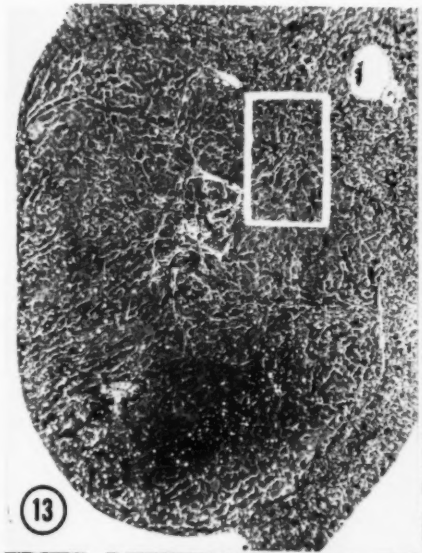
PLATE 81

FIGURE 13.—Small hepatoma in an intact male rat in experiment II, 10 weeks after beginning 2-diAAF administration. The tumor is composed of disorganized cords and sheets of large atypical hepatic cells with a minimum of supporting stroma and with scattered foci of necrosis and scarring. The adjacent liver is severely cirrhotic and contains a few small cysts. × 35

FIGURE 14.—Higher power of area marked off in figure 13. Note the loss of architecture, sparse stroma, and variability of the large cells comprising the tumor. The large size of the tumor cells can be better appreciated by comparing them with the non-neoplastic liver parenchymal cells trapped among the proliferating bile ducts and capillaries in the upper right corner of the photomicrograph. × 180

FIGURE 15.—A section of a large hepatoma composed of a disorderly growth of hepatic parenchymal cells which in places form pseudoacinar structures. This tumor was found in a castrated female rat given testosterone in experiment III, 8 months after beginning 2-diAAF administration. × 210

FIGURE 16.—Second-generation subcutaneous transplant of a large, poorly differentiated hepatoma of hepatic-cell type that was found in the liver of a castrated female rat given testosterone in experiment III, 7½ months after beginning 2-diAAF administration. This tumor was successfully transplanted subcutaneously for over 66 generations to other A × C rats. × 400



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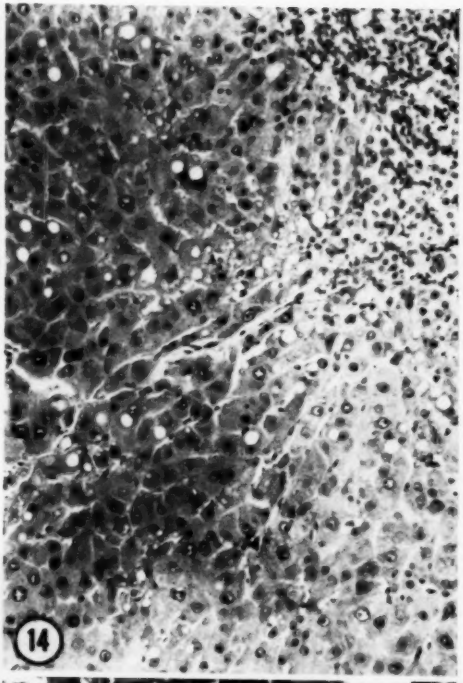

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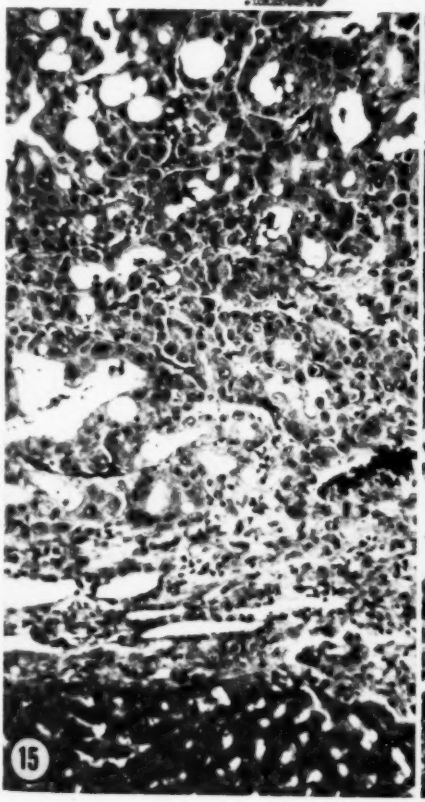

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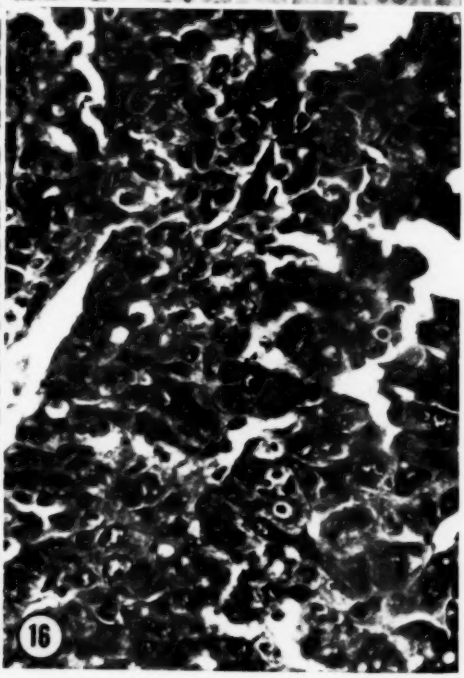

16

Morphologic Variations in an Osteogenic Sarcoma of the Mouse When Transplanted to the Kidney [1]

ALBERT W. HILBERG, *M.D., National Cancer Institute,*[2] *Bethesda, Md.*

Malignant tumors that become widely disseminated may show considerable morphologic variation in different sites of growth. The question arises whether this variation is due to a fundamental change in the character of the tumor or is simply a modification dependent upon the site of growth. Of particular importance may be modifications which suggest a greater degree of differentiation in a tumor, since this may indicate some loss of malignant characteristics. Transplantable animal tumors offer opportunities to study such modifications in tumor morphology as may be connected with the site of growth of the tumor.

An anaplastic osteogenic sarcoma in the mouse offered a good test object for the observation of alteration due to site of growth, since the formation of bone provides a readily observable manifestation of morphologic alterations in growth.

Materials and Methods

The osteogenic sarcoma arose at the distal end of the femur in a strain C3Hb/HeN female mouse, 34½ months of age, which had received no experimental treatment.[3]

Histologically, the original tumor had the features of a malignant osteogenic sarcoma similar to the one described by Barrett *et al.* (*1*). It was successfully transplanted and has been carried through 20 successive passages. During this period there was at first a gradual and later a rapid decrease in bone formation in the tumor along with a histologic change toward anaplasia and less differentiation. In the 6th to 20th generations, little or no bone tissue was identified.

Two fibrosarcomas were used as controls for studying bone-forming capacity in the transplantation experiments. One of these was a spontaneous fibrosarcoma arising in the lumbar region in a female C3Hb/HeN mouse, 26 months old.[4] The other fibrosarcoma arose in the left axillary region in a female mouse of the same strain at 16½ months of age that

[1] Received for publication August 26, 1955.

[2] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

[3] I am indebted to Dr. W. H. Eyestone, National Cancer Institute, for permission to use this tumor and for his valuable advice.

[4] Received from Dr. C. C. Congdon, National Cancer Institute.

had been given subcutaneous injections of benzidine technical (*2*) once a week for 45 weeks.[5]

Male and female strain C3Hb/HeN mice about 3 months of age were used for all procedures. Tissues from the osteogenic sarcoma and from the control fibrosarcomas were prepared by grinding in a glass tissue grinder until the suspension could be drawn into a 24-gauge needle. This suspension was then injected beneath the renal capsule, which was brought into view after an incision through the skin and subcutaneous tissues in the flank. Two tenths of a cc. of the suspension was injected under the renal capsule.

Thirty animals received injections of the osteogenic sarcoma beneath the renal capsule. Eleven animals were injected with the spontaneous fibrosarcoma and 5 with the induced fibrosarcoma. Most of the animals were killed in 90 to 180 days at the time they appeared moribund.

During the entire course of this experiment the spontaneous osteogenic sarcoma was continued in a parallel group of mice by subcutaneous transplantation. The tumor tissue was transplanted both by using an injection of tumor suspension identical to that used for subrenal-capsule transplantation and by using small pieces of tumor tissue which could be transplanted by means of a trocar. There was no instance, in these subcutaneous transplants, of a return of bone formation such as was observed in the animals receiving injections of tumor tissue beneath the renal capsule. The tumors transplanted in the subcutaneous site almost invariably grew to much greater size than those tumors resulting from the injections beneath the renal capsule. The subcutaneous tumors grew to sizes of 2 to 3 cm., whereas the largest of the tumors in the capsule area was 1 cm. in diameter.

Results

Thirteen of the animals which received transplants of the osteogenic sarcoma developed tumors beneath the renal capsule. The largest tumors extended through the capsule and measured from ½ to 1 cm. in diameter. The smallest tumor was 3 mm. in diameter. No difference in the histologic pattern could be detected in the tumors of different sizes. Six of the spontaneous fibrosarcoma transplants grew beneath the renal capsule. Four of the transplanted induced tumors grew. Tumors failed to grow in 12 animals injected with the osteogenic sarcoma and in 1 animal receiving the induced fibrosarcoma.

All the osteogenic sarcomas which grew beneath the renal capsule showed some bone formation. In some tumors the amount was slight, but in most there was an appreciable amount of bone formation. The location of the bone in the tumor varied. It was sometimes peripheral, but in the majority of cases the larger deposits of bone occurred centrally.

No hemorrhage, leukocytic infiltration, or fibrosis was found at the site of bone development, and it is therefore probable that the bone formation was not due to some irritant or trauma. Pulmonary metastases also

[5] Received from Dr. H. L. Stewart, National Cancer Institute.

showed bone formation. The fibrosarcoma transplants beneath the renal capsule showed no evidence of bone development. Subsequent subcutaneous transplants of the tumor tissue which grew in the renal areas and which produced bone grew in a manner similar to the parallel transplants of osteogenic sarcoma in the subcutaneous area. These tumors grew to a size of 2 to 3 cm. and in the first and second subcutaneous passages showed bone formation. Results from the third to the eighth passage were variable with respect to bone formation. In most animals bone failed to develop in the tumors.

In two animals the osteogenic sarcoma was accidentally placed in liver tissue. Bone was formed in the resulting tumor. In four animals fibrosarcoma was placed in the liver. No bone formation resulted.

Discussion

The experiment described was stimulated by the reports of LaCroix (*3*) and Huggins (*4,5*). Previous experiments have demonstrated the capacity of the renal capsule and tissue from the urinary tract to induce bone formation. LaCroix transplanted bone marrow beneath the renal capsule and produced bone for the purpose of studying early stages of development. Huggins showed that some factor present, in association with or elaborated by epithelium from various sites of the urinary system, was capable of causing bone production in tissue in contact with this epithelium. Both authors published extensive reviews concerning this phenomenon.

The present experiment showed that by transplanting an osteogenic sarcoma to a special site which had lost its bone-forming capacity after repeated subcutaneous transplantations, this capacity apparently was regained. The question yet remains whether the bone formed in the tumors growing beneath the renal capsule is developed by the tumor or by normal tissue as a reactive process. A distinction is not always easily made between bone formed by malignant osteoblasts and bone formed from normal tissue as a reaction to trauma, hemorrhage, or scarring. In favor of the opinion that the bone formed in transplants beneath the renal capsule is not reactive bone is the absence of signs of hemorrhage, either recent or remote, and the absence of inflammation or scarring adjacent to the site of bone formation. The bone spicules developing in the transplanted tumors were surrounded by tumor cells, and tumor cells could occasionally be demonstrated within the lacunae of the spicules of bone. It now appears that the tumor which arose from bone and which initially had the capacity to form bone still retained this capacity, although it had not been revealed during a long period of serial transplantation in the subcutaneous tissues. When the proper stimulus was provided, the anaplastic, undifferentiated osteogenic sarcoma again displayed this capacity to form bone. In two instances in which the tumor was transplanted to the liver, the ability to form bone also reappeared again.

When fibrosarcomas were transplanted beneath the renal capsule, no bone was formed. This lends support to the belief that the bone formed

in the osteogenic-sarcoma transplants was indeed neoplastic. It indicates that although there is a factor in the renal tissue which stimulates bone formation it is still necessary that neoplastic tissue transplanted to this site have the capacity, even though latent, to form bone.

Why the renal-capsule area or the liver potentiates bone formation is not known. It is possible that the high alkaline-phosphatase content of the renal and hepatic tissues, as shown by Gomori (*6*), McManus (*7*), and Longley and Fisher (*8*), may be a contributing factor.

Summary

An osteogenic sarcoma of the mouse became anaplastic and undifferentiated after numerous serial subcutaneous passages and no longer revealed any capacity for forming bone in this site. When it was transplanted into the renal-capsule area and liver, this capacity of bone formation was apparently regained.

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(*6*) Gomori, G.: Distribution of phosphatase in normal organs and tissues. J. Cell & Comp. Physiol. 17: 71–83, 1941.

(*7*) McManus, J. F. A.: Medical Diseases of the Kidney. Philadelphia, Lea & Febiger, 1950, 176 pp.

(*8*) Longley, J. B., and Fisher, E. R.: Alkaline phosphatase and the periodic acid Schiff reaction in the proximal tubule of the vertebrate kidney. Anat. Rec. 120: 1–21, 1954.

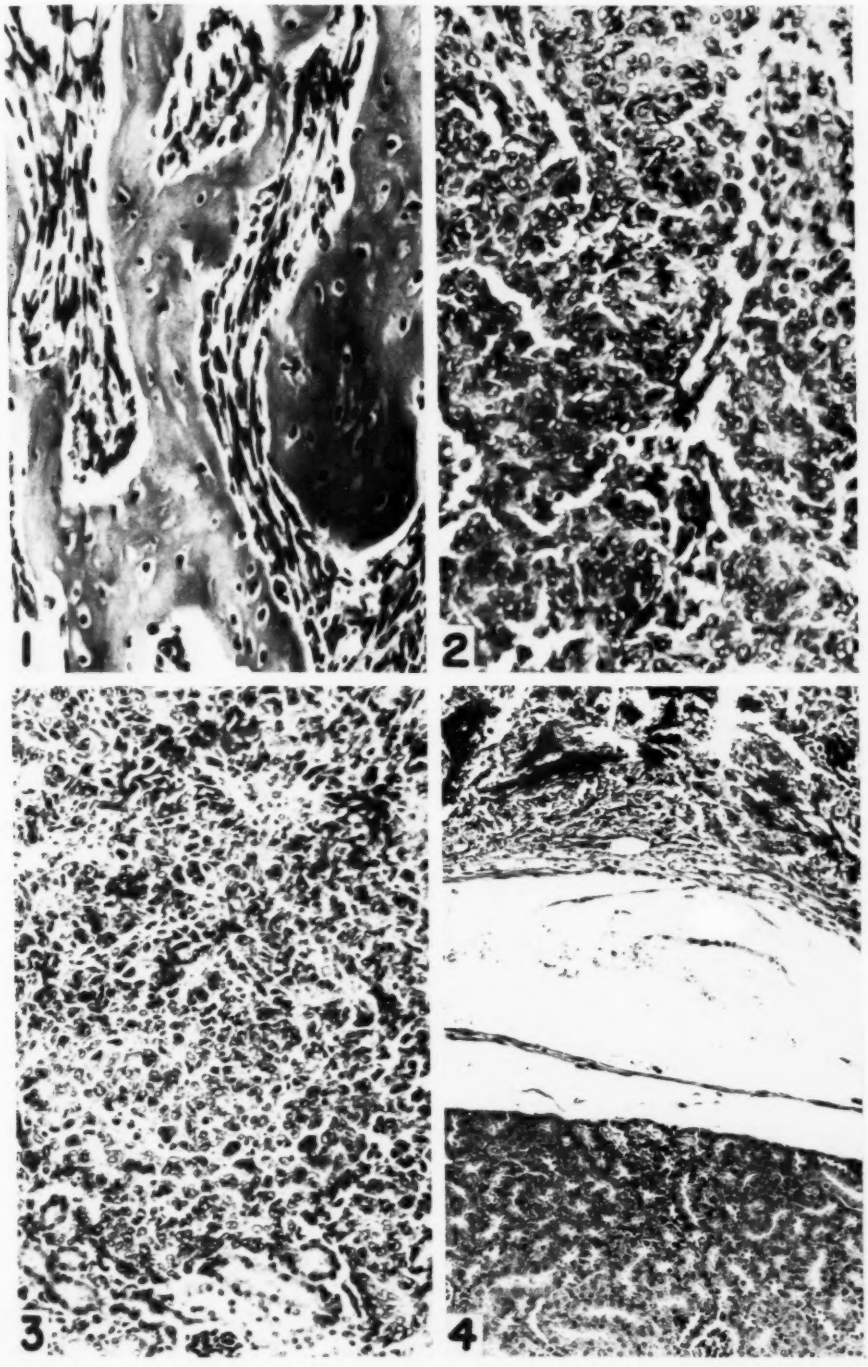
Plate 82

Figure 1.—Spontaneous osteogenic sarcoma. *Note* large trabeculae of neoplastic bone which show malignant osteoblasts surrounding the trabeculae and in the lacunae of the bone. × 275

Figure 2.—Osteogenic sarcoma in the seventh transplant generation in the subcutaneous area. *Note* absence of bone and characteristic colonnade pattern seen in transplanted osteogenic sarcomas of the mouse. × 250

Figure 3.—Renal-capsule transplant of osteogenic sarcoma, seventh generation. The renal tissue is seen in *lower left*. Above it is tumor tissue which shows bone formation. The bone shows as darker, rather homogeneous, areas within the tumor tissue. The colonnade architecture is maintained in this tumor. × 200

Figure 4.—Renal-capsule transplant of osteogenic sarcoma, seventh generation. Tumor spreading on the capsular surface shows extensive bone formation. × 125



Hilberg

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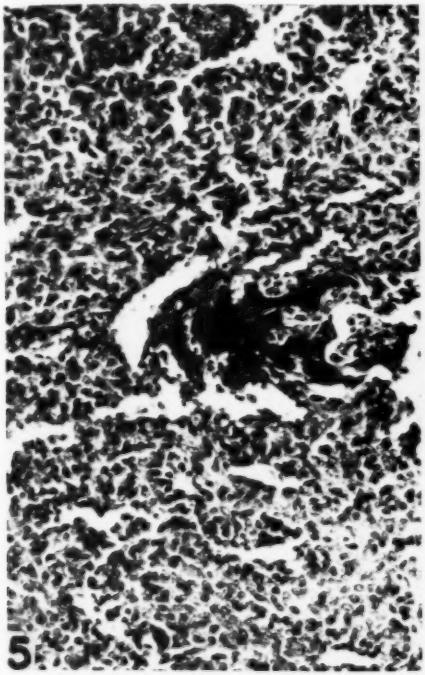
PLATE 83

FIGURE 5.—Intrarenal growth showing colonnade pattern. Bone formation is seen in the central portion of this tumor. × 200

FIGURE 6.—Renal transplant. Renal tissue in the *lower left* corner. The tumor shows bone formation but no change in the anaplastic character of the tumor cells. × 250

FIGURE 7.—Lung metastasis from renal transplanted tumor. *Note* the foci of bone formation. × 125

FIGURE 8.—Spontaneous fibrosarcoma transplanted to the renal-capsule area. No bone formation. The tumor grew well and locally invaded the kidney tissue. × 250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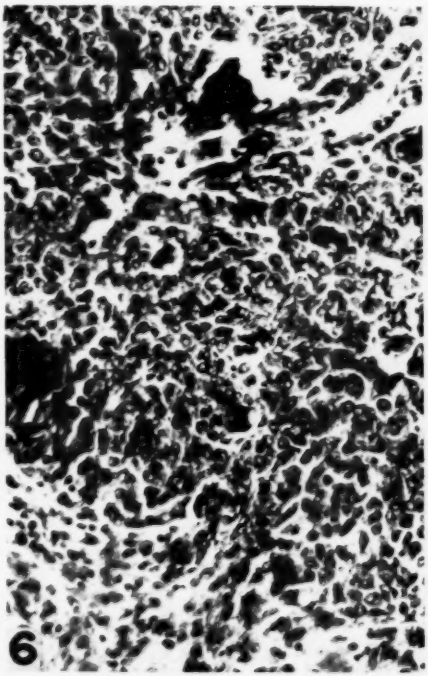
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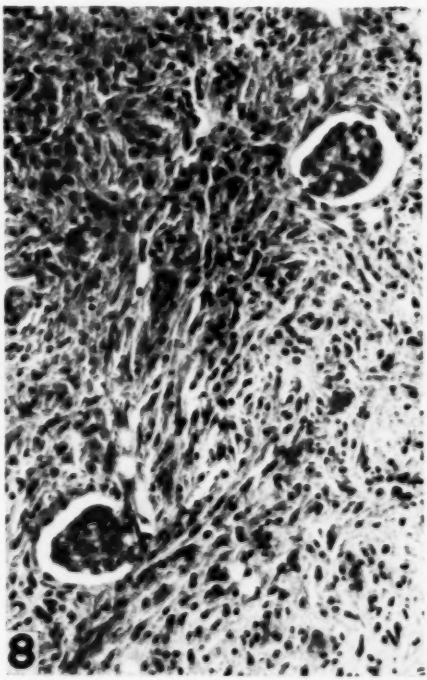
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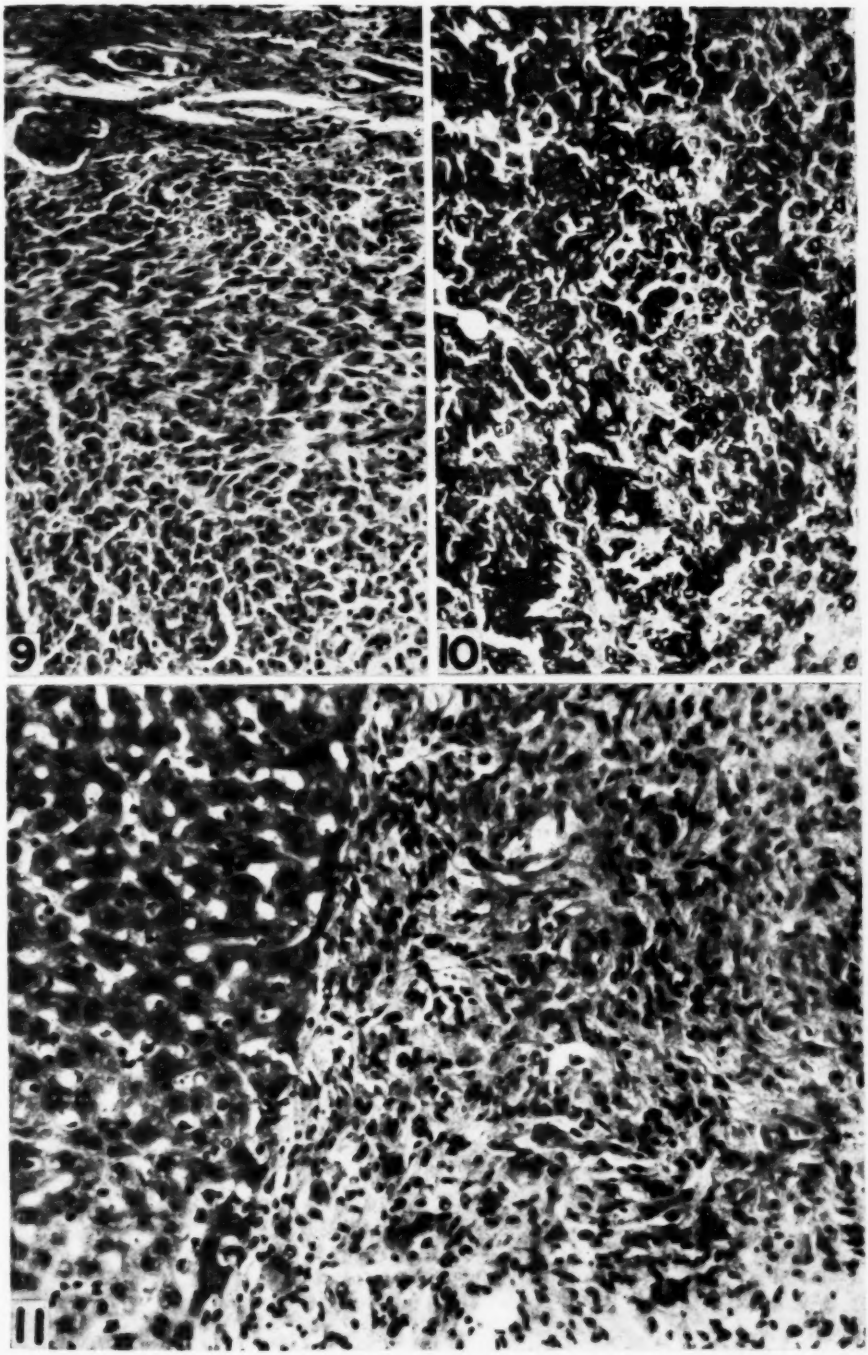
PLATE 84

FIGURE 9.—Induced fibrosarcoma transplanted in renal-capsule area. No bone formation. × 200

FIGURE 10.—Osteogenic sarcoma injected into liver. Liver tissue on the *right*. Considerable amount of bone formation is seen in the tumor tissue. × 250

FIGURE 11.—Fibrosarcoma injected into liver. Normal liver on the *left*, tumor on the *right*. No bone was formed by the tumor. × 250



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The Effects of Low Concentrations of Carcinogen in Epidermal Carcinogenesis. A Comparison with Promoting Agents [1, 2, 3]

UMBERTO SAFFIOTTI *and* PHILIPPE SHUBIK,[4] *Division of Oncology, The Chicago Medical School,*[5] *Chicago, Ill.*

The relationship of dose and response in carcinogenesis has been studied by many investigators using a variety of chemical and physical inducing agents (*1–6*). The present experiments are concerned with further studies of a similar nature and are specially designed to provide additional information on the two-stage theory of carcinogenesis (*7–13*). The investigation of tumors induced by a combination of a single subeffective dose of a carcinogenic hydrocarbon followed by repeated applications of croton oil has been used to demonstrate certain quantitative relationships in carcinogenesis otherwise not apparent. Thus, an alternative dose-response relationship of apparent biological significance can be obtained by a variation in test technique. When tumors are induced by repeated applications of a carcinogen only, both the number of tumors induced and the latent period will vary from carcinogen to carcinogen. This dual response makes quantitative assessment of the relative potencies of carcinogens difficult; for example, when 20-methylcholanthrene, 3,4-benzpyrene, and 1,2,5,6-dibenzanthracene are compared on the basis of the minimum quantity required to induce a tumor, they are found to be potent in the order DBA, MC, BP; whereas when compared on the basis of minimal latent periods of tumor induction, this order is changed to MC, BP, DBA (*2*). On the other hand, when a series of carcinogenic hydrocarbons are tested in single, subeffective applications, followed by croton oil, only the tumor incidence varies, the latent period remaining the same in all instances; it is hypothetically assumed that the tumor incidence in this circumstance can be taken to represent tumor-initiating potency, whereas the latent period is a measure of promoting potency (*10*).

In most of the experiments on the two-stage mechanism of carcinogenesis the vegetable mixture croton oil has been used as the promoting agent. It has been shown that the tumors induced in such experiments are

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[2] Supported by a cancer control grant (CS-0212) from the National Cancer Institute of the National Institutes of Health, U. S. Public Health Service.

[3] Part of this investigation has been presented at the 46th annual meeting of the American Association for Cancer Research, San Francisco, Calif., April 1955.

[4] With the technical assistance of Robert Feldman.

[5] 2755 West 15th Street, Chicago 8, Ill.

qualitatively different from those induced by repeated applications of a carcinogenic hydrocarbon in that many regress and most remain benign (*14*). Although many experiments are reported in which croton oil alone is noncarcinogenic (*5, 7–9, 13, 15–23*), recent reports have suggested that it may induce tumors by itself (*24, 25*). Should croton oil be a weak carcinogen, then a very low concentration of a known carcinogenic hydrocarbon should have similar effects, unless croton oil has some specific property besides carcinogenicity. In the present study, a low concentration of a carcinogenic hydrocarbon has been tested on both untreated and "initiated" mouse skin, and its action compared with that of croton oil.

Materials and Methods

The mice used in this investigation were 145 Swiss virgin females, 2 to 3 months old at the beginning of the experiments. They were obtained from the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine, and bred in this laboratory. They were housed in plastic cages and fed Rockland mouse diet with water *ad libitum*.

The carcinogen used was 9,10-dimethyl-1,2-benzanthracene (DMBA) (Eastman Organic Chemicals) in mineral oil (Superla 34, Standard Oil of Indiana) at concentrations of 1 and of 0.01 percent. The promoting agent was croton oil (Boots, B. P.) as a 5 percent solution in mineral oil.

All solutions were painted on an area 1.5 cm. square of the skin of the interscapular region, which was clipped free of hair with small scissors. Each application consisted of 1 drop of approximately 25 λ from a glass dropper.

The initiating treatment consisted of a single painting with the 1 percent solution of DMBA. After an interval of 1 week, the subsequent paintings followed twice weekly throughout the experiment. Each painting with the 1 percent of DMBA corresponded to approximately 250 γ of DMBA, and each painting at 0.01 percent to approximately 2.5 γ of DMBA.

The mice were divided into 5 groups:

Group 1: Received a single application of 1 percent DMBA followed by paintings twice weekly with the 0.01 percent solution of DMBA.

Group 2: Received no initiating treatment; was only painted twice weekly with the 0.01 percent solution of DMBA.

Group 3: Received only the single application of 1 percent DMBA and no further treatment.

Group 4: Received a single application of 1 percent DMBA and then paintings twice weekly with croton oil.

Group 5: Received no initiating treatment; and was only painted twice weekly with croton oil.

Each mouse was numbered by ear clipping and, as the tumors started to appear, examined weekly and its tumors were charted. The charts, as described in a previous paper (*26*), record the duration, size, and morphologic type of each tumor. At the death of the animal, histologic confirmation was obtained. For the purpose of the present investigation the tumors are described as papillomas, carcinomas, and regressions, the latter repre-

senting papillomas that regressed and disappeared after a certain time (tumors that regressed before 4 weeks from the time of first appearance were not considered but were discarded from the counts).

Results

The results are summarized in table 1. The duration of the experiments was 62 weeks for all groups, except for group 4 which was interrupted after 25 weeks.

The following observations can be made:

1) A concentration of 0.01 percent DMBA in mineral oil is effective when repeatedly painted for a long period of time, the latent period for the tumors averaging 50 weeks.

2) When a low concentration of DMBA is painted on a previously initiated tissue, the average latent period is shortened, but the induction of tumors follows the same pattern. From table 1, one can calculate that the number of tumors per tumor-bearing mouse is 2.7 for both groups 1 and 2; and the same can be said for the number of carcinomas per tumor-bearing mouse—1.4 in group 1 and 1.3 in group 2.

3) A more detailed analysis is shown in a graphic presentation of the results in groups 1 and 2 (text-fig. 1, chart 1). The curves for the total number of tumors, as well as those for malignancies, follow the same pattern in the two groups, with an interval shift of about 10 weeks between the curves for each group. This leads to a consideration of dosage effects. The animals in group 1 received a single painting of 1 percent DMBA and subsequent paintings of 0.01 percent DMBA twice weekly. It took 100 paintings (in 50 weeks) for the animals of group 2, to receive the same amount of carcinogen that was put on the first group all at once with the single painting of 1 percent DMBA. This amount of carcinogen, applied repeatedly at a low concentration over a long time, was an effective carcinogenic treatment, while the same amount of carcinogen, applied at one time with no other treatment (group 3), did not yield any tumors.

4) The single painting of 1 percent DMBA did not produce tumors on its own, but, when followed by croton oil, induced a great number of tumors rapidly, with many regressions and very few malignancies (group 4, text-fig. 1, chart 2). Croton oil by itself did not produce tumors.

5) A further comparison between charts 1 and 2 shows a different biological behavior of the induced tumors in the two groups painted with a low concentration of carcinogen on the one hand (chart 1) and the two groups painted with a promoting agent on the other (chart 2). First, repeated applications of the solution containing 0.01 percent DMBA were able to induce tumors without prior treatment, with a latent period relatively only a little longer than that with the same treatment preceded by a single painting of 1 percent DMBA. The promoting agent by itself does not produce tumors (group 5). The second major difference concerns the morphologic type of tumors produced. Repeated applications of a low concentration of carcinogen yield a number of tumors, many of

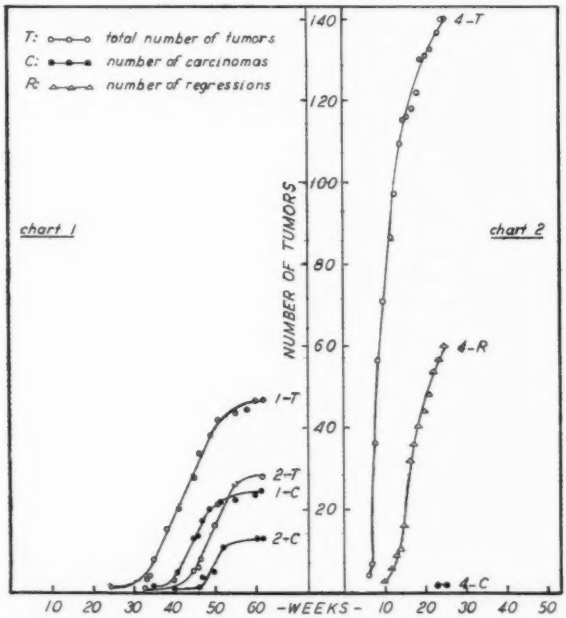
TABLE 1.—*Distribution pattern of the induced tumors*

Group	Treatment*†		Initial number of mice	Survivors at time of first tumor	Number of tumor-bearing mice	Total number of tumors	Number of carcinomas	Number of regressions	Average latent period (*weeks*)‡
	Single painting	Paintings twice weekly							
1	1% DMBA	0.01% DMBA	30	27	17	47	24	—	43
2	—	0.01% DMBA	20	18	10	27	13	—	50
3	1% DMBA	—	50	40§	—	—	—	—	—
4	1% DMBA	5% croton oil	25	23‖	14	140	2	60	12
5	—	5% croton oil	30	15¶	—	—	—	—	—

*All solutions in mineral oil.
†One-week interval between the initiating single application and the beginning of the following paintings.
‡Calculated for all the tumors produced.
§Survivors after 52 weeks; observations continued until the 62nd week.
‖Terminated at 25 weeks.
¶Survivors after 37 weeks; 8 survivors after 52 weeks—treatment continued until the 62nd week.

which appear *de novo* as malignancies or soon turn into malignancies (51.0% in group 1 and 48.1% in group 2). In these groups in no instance was a tumor seen to regress. On the contrary, the group painted with the promoting agent after initiation (group 4) rapidly produced a large number of tumors that were mostly benign and had a high rate of regression (42.8%).

This group, in which tumors were elicited by croton oil, continued for only 25 weeks as compared with 62 weeks in the groups in which carcinogen alone was used. Therefore, it may be said that a comparison between



TEXT-FIGURE 1.—Patterns of tumor induction. *Chart 1.*—Group 1 (curves 1-T and 1-C): 1 percent DMBA painted once, followed by 0.01 percent DMBA twice weekly; Group 2 (curves 2-T and 2-C): 0.01 percent DMBA painted twice weekly. *Chart 2.*—Group 4 (curves 4-T, 4-R, and 4-C): 1 percent DMBA painted once, followed by 5 percent croton oil twice weekly.

the types of tumor seen under these two conditions is not strictly possible. However, it can be seen that croton-oil promotion gave rise to a large number of benign tumors with great rapidity. The tumors induced by low concentrations of the carcinogen did not arise until very late in this study, and the comparison of morphologic type has been made on the basis of the length of time tumors were present rather than upon the length of treatment involved. It would seem clear that, given comparable periods in the life history of the individual tumors, those elicited with croton oil are comparatively far more benign than those induced with the carcinogen alone.

Discussion

Certain investigators have realized the necessity for a better understanding of dose-effect relationships in carcinogenesis in order to study such vital questions as tolerance levels. In particular, the work of Druckrey (*3*) has demonstrated the irreversibility of carcinogenic action and its proportionality to the total quantity of carcinogen administered. Both Druckrey (*3*) and Graffi (*4,5*) have demonstrated a "summation effect" when a carcinogen is administered in repeated low doses. According to these investigators, there is no threshold dose of a carcinogen that can be considered harmless, since these agents have a cumulative effect and the minutest dose given often enough will result in tumors. The studies reported here demonstrate that total dosage alone is, under certain conditions, only one of the factors governing the response in carcinogenesis, as suggested by Graffi (*5*); it has been found that 250 γ of DMBA applied once only at 1 percent concentration will not give rise to skin tumors, whereas a somewhat smaller amount, when divided into a series of 2.5 γ doses at 0.01 percent and administered over a very long time, will give rise to tumors. The exact relationship of quantity of carcinogen administered to tumors induced is clearly a complex matter; no direct correlation seems possible under all conditions; for the time being it would seem that small, repeated doses are more effective than large, single applications, irrespective of total quantity.

The second aspect of the experiments reported refers to the role played by promoting action in the quantitative interpretation of carcinogenesis. Numerous experiments, mostly concerned with skin carcinogenesis, have lent support to the view that a two-stage mechanism is involved; thus carcinogenesis, it is suggested, is begun by the initiation of some normal cells to latent tumor cells that are subsequently converted into tumors by promoting action; not only can carcinogens promote, but this process, unlike initiation, can be subserved by noncarcinogenic agents such as croton oil, wound healing, and others (*12, 27–32*). According to this hypothesis, the carcinogenic hydrocarbon applied to the skin is producing a dual effect. The present experiments were designed to see if the two effects of the hydrocarbon carcinogen could be distinguished in an experiment using no other promoting agent. A low dose of hydrocarbon was tested repeatedly both on the skin of a normal mouse and on the skin of a mouse "initiated" with a single larger dose of carcinogen. It was found that a summation effect occurred only to a certain degree and that the hydrocarbon at low dosage, when painted on pretreated skin, induced tumors some 10 weeks sooner.

Many investigators have tested croton oil alone repeatedly and reported either no tumors or an occasional papilloma in relatively large groups (*5, 7–9, 13, 15–23*). In recent years some investigators have suggested that this promoting agent itself may be carcinogenic (*24,25*). In the present experiments, the action of croton oil following a single dose of carcinogen has been compared with the effect of a low concentration of carcinogen used for the same purpose. If croton oil has no special prop-

erties as a promoting agent, and is merely, as has been suggested, a carcinogen, then the tumor patterns emerging should be essentially the same in both cases. However, as can be seen from the results, the croton-oil promotion induced more tumors, and far more rapidly, than the low concentration of carcinogen used in the same way. Furthermore, the tumors induced with the croton oil were largely benign and showed a high proportion of regressions; those induced by the low concentration of carcinogen were mostly malignant and none regressed. This low concentration of carcinogen, painted without prior treatment, induced a high proportion of tumors; the croton oil alone induced none. Since the majority of reports in the literature suggest that croton oil is essentially noncarcinogenic, it would seem that the few reports to the contrary should be carefully examined. Since it seems clear that croton oil has a powerful promoting action, minimal contamination with carcinogen could result in a false positive. The possibility that organic solvents used may contain trace amounts of carcinogen must always be considered. The recent report by Selye (*25*) that croton oil induces sarcomas in the rat merely records the induction of sarcomas in 5 rats in which "granuloma pouches" were produced with the aid of croton oil in corn oil. No control group is included in this study, and for the time being this result must be classified as interesting but it does not demonstrate a particular connection between any single one of the factors involved and carcinogenesis. It should be noted that Klein found no sarcomas following injection of croton oil in the mouse under proper experimental conditions (*16,18*) and that no effects were noted with this substance painted on the skin of the rat (*31*).

It seems clear that even if croton oil may be demonstrated to have a weak carcinogenic action, it possesses, in addition, a very specific promoting property. In order to assess carcinogenesis quantitatively many factors must be taken into account, and, among these, the possibility of a two-stage process is still important.

The present experiments concern only a particular instance of carcinogenesis. It would seem worth while studying the action of other carcinogens under similar circumstances. The methodology reported here also may be of some importance in evaluating carcinogenicity tests for environmental hazards and in distinguishing the action of traces of carcinogen from that of a promoting agent in a mixture of unknown constitution.

Summary

1) The effects of repeated skin paintings with low concentrations of carcinogen on normal mice and on mice previously initiated with a single subeffective painting of the same carcinogen are studied, and a comparison is made under the same conditions with the effects of the promoting agent croton oil.

2) The pretreatment with a single application of a 1 percent solution of 9,10-dimethyl-1,2-benzanthracene in mineral oil, insufficient to produce tumors unaided, shortened the latent period for the appearance of the tumors brought out by the repeated applications of the 0.01 percent

solution of the same carcinogen. The dose-response relationship is analyzed.

3) The pattern of tumor induction is similar in the two groups painted repeatedly with the carcinogen in low concentration, but differs substantially from that of the group treated with a single application of carcinogen followed by croton oil. This again emphasizes the specificity of action of croton oil and distinguishes it clearly from activity that may be attributable to weak carcinogenic action.

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On the So-Called "Thiamin-Destructive Influence" by the Saliva of Patients with Cancer or Other Disease [1]

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In 1942 Fujita (*1*) found an enzyme in certain shellfish which he named "aneurinase." He discovered that this enzyme decomposes thiamin. Among shellfish, the enzyme was found to be most active in clams and related shellfish.

In America studies on the destruction of thiamin by tissues from fish were published by Woolley (*2*), Spitzer *et al.* (*3*), and Deutsch and Ott (*4*) about 1941. Sealock *et al.* (*5*) reported the presence of a "thiamin destructive factor" in the tissue of carp in 1943. The enzyme was found to be active only in fresh-water fish. In 1944 Krampitz and Woolley (*6*) prepared this enzyme from fresh-water fish and named it "thiaminase."

In 1948 Shibata and Chang (*7, 8*) noted that the feces of a patient with habitual constipation and thiamin deficiency contained thiaminase, and in 1949 Matsukawa and Misawa (*9*) isolated a thiaminase-producing organism from feces that contained thiaminase.

Thus, at present, there are three known sources of thiaminase: shellfish, fresh-water fish, and bacteria. On the other hand, studies of thiamin-destroying agents in warm-blooded animals seem to be very rare.

It was postulated by Muckenfuss (*10*) in 1918 that the amount of thiamin in human saliva is very small. This assumption was later confirmed by Glavind *et al.* (*11*) and by Shibata (*12*). Glavind's study indicated that the amount present was 0.7γ per 100 ml.; Shibata reported that total thiamin was about 2.18γ per 100 ml. and free thiamin was not found.

Miura *et al.* (*13*), on the other hand, noted thiochrome-negative influence of thiamin by human saliva, *i.e.*, human saliva converts thiamin into a substance that cannot be oxidized to thiochrome (see *14–16*).

These data were based on studies using the saliva of a normal person, and no description was given on the relationship to thiamin in the saliva of abnormal subjects. Furthermore, no comparison was made of the amount of thiamin in saliva with that in the salivary glands of the same subjects.

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Materials and Methods

The mouths of patients were rinsed clean with a 0.03 *N* hydrochloric-acid solution, and the saliva obtained during the next 5 minutes was analyzed for thiamin by the method described below. The same method of analysis was used for the saliva of dogs, obtained by injecting the animals subcutaneously with 0.3 ml. of a 1 percent pilocarpine solution and collecting the saliva secreted.

The thiochrome method (*14–16*) was utilized for the determination of thiamin. In those experiments in which the thiamin level was being measured, the following special precaution was employed: the saliva, immediately after it was obtained, was adjusted to *p*H 4.5 with 0.1 *N* HCl and submerged into boiling water for 20 minutes in order to preserve as much thiamin as possible.

In measuring the enzymatic activity of saliva for its thiamin-destruction ratio, 1 ml. of saliva was reacted with 2γ of thiamin at 60° C. for 1 hour, *p*H 6.5, using 0.1 *M* citric acid buffer in a volume of 18 ml. The ratio was then calculated from the disappearance of thiamin, as indicated in table 1. After the volume was made to 20 ml., as indicated in table 1, a 4 ml. aliquot was used for the thiamin determination. The 4 ml. aliquot was dephosphorylated by Takadiastase by adding 1 cc. of a Takadiastase extract after addition of a drop of toluene. The mixture was incubated at 37° C. for 1 hour. After this incubation, 4 ml. aliquots were used for the measurement of thiamin by the thiochrome method. As a control for the determination of the amount of thiamin originally present, the procedure was carried through using saliva that had been heated to 100° C. at *p*H 4.5 for 20 minutes. A control tube with heated saliva was always run along with a control tube containing unheated saliva in each set of determinations (see table 1 for protocol).

The amount of free thiamin was determined, following its absorption on permutit, by a modification of the thiochrome method. The permutit

TABLE 1.—*Method of measuring thiamin-destruction ratio**

Constituents	Blank (*ml.*)	Experimental (*ml.*)	Heated control (*ml.*)	Unheated control (*ml.*)
Saliva	1	1	1†	1
Water	12	10	10	12
Buffer (*p*H 6.5)	5	5	5	5
Thiamin (1γ per cc.)	—	2	2	—
After adding 1 drop of toluene, heat at 60° C. for 1 hour; then make to 20 ml. by adding:				
Thiamin (1γ per cc.)	—	—	—	2
Water	2	2	2	—
Add 1 cc. of extract of Takadiastase to 4 ml. aliquots.				
Add 1 drop of toluene and keep at 37° C. for 1 hour.				
Take 4 ml. from each tube and measure thiamin by thiochrome method.				

*Thiamin-destruction ratio percent $= \frac{\text{Control} - \text{Experimental}}{\text{Control} - \text{Blank}} \times 100$

†Heat at 100° C. (*p*H 4.5) for 20 minutes.

was first treated as described by Hennessy and Cerecedo (*14*). After the solution was passed through the permutit column, the column was washed 2 or 3 times with 10 ml. of water, and the absorbed thiamin was washed out with 15 ml. of a boiling solution of 25 percent KCl in 0.1 *N* HCl. A mixture of 0.1 ml. of 1 percent potassium ferricyanide and 3.0 ml. of 30 percent NaOH was added to the eluted thiamin, and the whole was vigorously shaken with 8 ml. of butanol. After centrifugation, the upper layer was transferred to a test tube containing 1 gm. of anhydrous sodium sulfate, and the mixture was well shaken. Five ml. of the clear supernatant was used for the fluorometric determination of thiochrome.

Experimental Results

This investigation is the first to appear in the literature on the amount of thiamin present in both the saliva and salivary glands of normal dogs. A summary of the data is presented in table 2. The amount of thiamin in the parotid glands of five of the dogs averaged 15.0γ of total thiamin per 100 gm. and 2.4γ of free thiamin per 100 gm. In the maxillary glands of three of the dogs the total thiamin was 57.1γ per 100 gm. and the free thiamin was 7.2γ per 100 gm. The amount in the saliva was found to be much less than that in the salivary glands; it averaged 3.9γ per 100 ml. of total and 0γ per 100 ml. of free thiamin. For the above experiments 5 ml. of saliva was used. However, we found it impossible to find evidence of thiamin destruction *in vitro* using saliva from dogs.

In the saliva of 15 human beings total thiamin, measured in 5 ml. of saliva, averaged 7.7γ per 100 ml. and in 7 human beings free thiamin, measured in 50 ml. of saliva, averaged 0.3γ per 100 ml. The data appear in table 3. The values obtained were higher than those in previous reports, probably because the saliva, immediately after it was obtained, was adjusted to *p*H 4.5 and treated in boiling water. When saliva samples from each of 6 human beings were kept at room temperature after collection, the average amount of thiamin decreased to 6.4γ per 100 ml. after 15 minutes and to 4.9γ per 100 ml. after 30 minutes. However, the

TABLE 2.—*Amount of thiamin in salivary glands and saliva of dogs*

Parotid glands			Maxillary glands			Saliva		
Dog	Total thiamin (γ/100 gm.)	Free thiamin (γ/100 gm.)	Dog	Total thiamin (γ/100 gm.)	Free thiamin (γ/100 gm.)	Dog	Total thiamin (γ/100 ml.)	Free thiamin (γ/100 ml.)
1	11.2	0.0	1	56.3	0.0	1	1.1	0.0
2	11.2	3.7	2	56.3	5.6	2	1.1	.0
3	22.5	3.6	3	67.5	11.3	3	4.5	.0
			4	50.6	5.6	4	9.0	.0
			5	78.8	11.3			
			6	33.8	11.3			
			7	56.3	5.6			
Average	15.0	2.4		57.1	7.2		3.9	.0

TABLE 3.—*Amount of thiamin in normal human saliva**

Measured in 5 ml. of saliva			Measured in 50 ml. of saliva	
No.	Total thiamin (γ/100 ml.)	Free thiamin (γ/100 ml.)	No.	Free thiamin (γ/100 ml.)
1	6.3	0.0	1	0.1
2	13.5	.0	2	.5
3	12.3	.0	3	.2
4	13.5	.0	4	.5
5	5.6	.0	5	.1
6	9.0	.0	6	.2
7	3.4	.0	7	.2
8	2.3	.0		
9	5.6	.0		
10	2.3	.0		
11	5.6	.0		
12	5.0	.0		
13	7.2	.0		
14	13.5	.0		
15	11.3	.0		
Average	7.7	.0		.3

*Immediately after obtained, adjusted to pH 4.5 and treated for 20 minutes with boiling water.

saliva which was immediately treated with boiling water averaged 8.3γ per 100 ml. (table 4).

An estimation of the thiamin in the saliva of 40 normal human beings indicated that the destruction ratio averaged 37 percent (table 5).

In addition, an investigation was made to determine how the thiamin-destructive influence changes in patients who had such malignant tumors as cancers of the stomach, tongue, esophagus, liver, and mammary gland and reticulosarcoma. Not only was the destruction ratio decreased in these patients (average = 8%), but in most cases no destruction took place (table 6). In spite of this, in the case of the patients with cancer of the digestive organs, *e.g.*, gastric or pancreatic cancer, the amount of thiamin in the saliva was less than in normal people (table 7).

Since the thiamin level in the saliva of patients with cancer of the gastrointestinal tract is low, while that of patients with other types of

TABLE 4.—*Differences in amount of thiamin in saliva left at room temperature (15 to 20° C.)*

No.	Immediately after obtained (γ/100 ml.)	After 15 minutes (γ/100 ml.)	After 30 minutes (γ/100 ml.)
1	7.2	5.6	5.0
2	6.3	5.0	1.1
3	13.5	9.0	9.0
4	11.3	11.3	9.0
5	5.0	1.1	0.0
6	6.3	6.3	5.0
Average	8.3	6.4	4.9

TABLE 5.—*Thiamin-destruction ratio in normal human saliva*

No.	Destruction ratio (*percent*)	No.	Destruction ratio (*percent*)
1	65	22	25
2	67	23	20
3	41	24	0
4	60	25	30
5	40	26	50
6	40	27	33
7	42	28	30
8	50	29	50
9	40	30	35
10	0	31	33
11	33	32	60
12	16	33	28
13	28	34	66
14	0	35	33
15	42	36	33
16	50	37	25
17	39	38	0
18	58	39	40
19	50	40	30
20	50		
21	33	Average	37

cancer is higher than these cases, one might postulate that the lower value in the former group might be due to insufficient intake of food. This lower value is present even though there is a decreased thiamin-destruction ratio.

The same phenomenon was observed in patients with serious liver insufficiency due to liver cirrhosis or cholecystitis, *i.e.*, the amount of thiamin was less than normal in these cases despite the lowered thiamin-destruction ratio (tables 8 and 9).

In all series of determinations of saliva from patients, a determination on normal saliva was included. In a study of several patients with other diseases, those patients with gastritis, gastric ulcer, and pulmonary tuberculosis gave values that were nearly normal (table 10).

Discussion

As we have reported previously, the thiamin-destruction ratio in tissues is increased in animals with cancer (*19*) or with liver insufficiency produced by carbon tetrachloride (*20*) compared with the ratio in normal animals (*20*); but, as shown in this paper, the relationships are somewhat different when the values for saliva are compared. The destruction ratio for saliva is decreased in animals with cancer or liver insufficiency. Such variation suggests that in all warm-blooded animals there exist some (two or more) mechanisms by which thiamin is destroyed and that most of the destruction is probably carried out by tissues since the influence of saliva appears to be very weak.

TABLE 6.—*Thiamin-destruction ratio in patients with malignant tumor*

No.	Diagnosis	Destruction ratio (*percent*)	No.	Diagnosis	Destruction ratio (*percent*)
1	Gastric cancer	0	19	Tongue cancer	0
2	Pyloric cancer	0	20	Reticulosarcoma	25
3	Gastric cancer	0	21	Reticulosarcoma	0
4	Cardiac cancer	0	22	Reticulosarcoma	0
5	Gastric cancer	16	23	Widespread carcinoma of peritoneal cavity	12
6	Gastric cancer	15			
7	Pyloric cancer	20	24	Liver cancer	0
8	Gastric cancer	0	25	Liver cancer	0
9	Gastric cancer	22	26	Liver cancer	0
10	Gastric cancer	0	27	Mammary cancer	22
11	Gastric cancer	0	28	Mammary cancer	5
12	Gastric cancer	0	29	Mammary cancer	18
13	Esophageal cancer	20	30	Mammary cancer	10
14	Esophageal cancer	20	31	Lung cancer	15
15	Esophageal cancer	9	32	Lung cancer	10
16	Pancreatic cancer	0			
17	Bronchial cancer	30			
18	Tongue cancer	0	Average		8

TABLE 7.—*Amount of thiamin in saliva of patients with malignant tumor*

No.	Diagnosis	Total thiamin (γ/100 ml.)	No.	Diagnosis	Total thiamin (γ/100 ml.)
1	Gastric cancer	3. 8	14	Lung cancer	6. 8
2	Gastric cancer	6. 0	15	Lung cancer	2. 3
3	Gastric cancer	1. 5	16	Lung cancer	3. 8
4	Gastric cancer	2. 3	17	Lung cancer	3. 8
5	Gastric cancer	1. 2	18	Lung cancer	3. 0
6	Gastric cancer	1. 1	19	Mammary cancer	4. 5
7	Gastric cancer	1. 1	20	Mammary cancer	7. 8
8	Gastric cancer	1. 1	21	Mammary cancer	4. 5
9	Gastric cancer	2. 3			
10	Gastric cancer	3. 4			
11	Gastric cancer	4. 5			
12	Pancreatic cancer	2. 3			
13	Pancreatic cancer	0. 9			
Average		2. 4	Average		4. 6

TABLE 8.—*Thiamin-destruction ratio in patients with liver insufficiency*

No.	Diagnosis	Destruction ratio (*percent*)	Millon's value* (*mg./100 ml.*)	Bromsulphalein test (*percent*)	Thymol turbidity test (*M. unit*) †	Takata reaction (*18*)
1	Liver cirrhosis	0	33. 5	24	10. 0	++++
2	Liver cirrhosis	22	5. 0	10	5. 0	−
3	Liver cirrhosis	0	44. 5	32	13. 2	++
4	Liver cirrhosis	15				
5	Liver cirrhosis	28	22. 5	20	8. 0	++++
6	Cholecystitis	0				
7	Cholecystitis	10				
Average		9				

*Imanaga's modification of Millon's reaction (*17*).
†M. unit = Muclagan unit.

TABLE 9.—*Amount of thiamin in saliva of patients with liver disease*

No.	Diagnosis	Total thiamin (γ/*100 ml.*)	Millon's value* (*mg./100 ml.*)	Bromsulphalein test (*percent*)	Thymol turbidity test (*M. unit*) †	Takata reaction (*18*)
1	Liver cirrhosis	2. 3	15			
2	Liver cirrhosis	3. 3	36	32	7. 6	++++
3	Liver cirrhosis	4. 5	32	6	7. 0	−
4	Liver cirrhosis	3. 7	19	21	1. 4	−
5	Liver cirrhosis	1. 8	41	21	1. 1	±
6	Liver cirrhosis	2. 3	24	13	1. 9	−
7	Liver cirrhosis	1. 8	26	34	4. 5	++
8	Liver cirrhosis	2. 3				
Average		2. 5				

*Imanaga's modification of Millon's reaction (*17*).
†M. unit = Muclagan unit.

TABLE 10.—*Thiamin-destruction ratio in patients with other disease*

No.	Diagnosis	Destruction ratio (*percent*)
1	Gastric ulcer	9
2	Gastric ulcer	40
3	Gastroptosis	30
4	Exophthalmic goiter	0
5	Adenomatous goiter	37
6	Pulmonary tuberculosis	10
7	Pulmonary tuberculosis	33
8	Pulmonary tuberculosis	0
9	Pulmonary tuberculosis	30
10	Pulmonary tuberculosis	20
11	Pulmonary tuberculosis	27
12	Tuberculous lymphadenopathy in the neck	50
13	Hemorrhoid	22

Summary

1) The amount of thiamin in the salivary glands was compared with that in the saliva of the same animal (dog); it was noted that the latter contained much less thiamin than the former.

2) The amount of thiamin in human saliva was also reinvestigated.

3) The thiamin-destruction ratio by normal human saliva averaged 37 percent, but this average was smaller in the saliva of patients with cancer or liver insufficiency.

4) In the case of patients with cancer of the digestive organs, the amount of thiamin in the saliva was less than in normal people despite a lowered thiamin-destruction ratio. This may be due to their poor state of nutrition.

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The Effect of Bacitracin on Sunflower Tissue Cultures Contaminated with Bacteria [1, 2]

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One of the risks during the routine experimentation and maintenance of plant tissues growing *in vitro* is contamination. Among sunflower-callus tissue cultures grown *in vitro* in this laboratory, about 50 percent or more revealed bacterial contamination. The present study was undertaken in order to develop a method for alleviation of this problem.

Bacteria growing in competition with the tissue are undesirable and must be eliminated through either physical or chemical means. In addition to a thorough review and a re-evaluation of the aseptic techniques used in this work, the use of antibiotics was considered as a possible measure to control bacterial contamination. The antibiotic(s) selected must be convenient to use, and it is desirable that it not alter the established procedures or affect the experimental results. It is preferable that the antibiotic inhibit only the growth of the contaminating organism without disturbing conditions which are optimal for the growth of sunflower tissue. In other words: 1) It must withstand autoclaving, since it is undesirable from the standpoint of aseptic technique to pipette substances into hundreds of tubes. 2) It must function in the presence of the normally supplied nutrients and tissue exudates in the medium. 3) The substance(s) chosen must not alter the growth or the normal physiological patterns of the tissues.

A search was made for an antibiotic substance which would fulfill the above requirements. Bacitracin was finally selected to control this bacterial contaminant in sunflower-callus tissue cultures. The question of whether the organism is specific for the tissue or a "chance" contaminant is not essential to this study. It is felt that the procedures outlined here rather than the results are the real substance of the paper, since they may be helpful to others who have been or may be faced with a similar situation.

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[2] This work was supported jointly by research grants-in-aid from the American Cancer Society upon recommendation of the Committee on Growth of the National Research Council, and by research grant C-1632 from the National Cancer Institute of the National Institutes of Health, U. S. Public Health Service.

[3] The authors are grateful to Dr. C. M. Ford, of the Department of Bacteriology of Tuskegee Institute and The Carver Foundation, for his technical advice and helpful suggestions from time to time regarding certain aspects of the work and for his critical appraisal of the paper.

Materials

The antibiotics (streptomycin-SO4, bacitracin, and penicillin) used in these experiments were made available by Chas. Pfizer & Co., Inc., Brooklyn, New York. Aureomycin-HCl was obtained from the Lederle Laboratories Division, American Cyanamid Company, Pearl River, New York. The sunflower-callus tissue cultures were initiated in the laboratories of the California Institute of Technology during 1948 (*1*).

Methods, Results, and Discussion

Assay of antibiotics.—The disc technique was employed to ascertain the antibiotic best suited for control of the contaminating organism *Bacillus* sp.[4] Aureomycin, bacitracin, penicillin, and streptomycin were dissolved in glass-distilled water, and for each antibiotic solution a final concentration of 5 ppm was obtained. The solutions were autoclaved for 15 minutes at 20 pounds pressure.[5] Sterile filter-paper discs (12 mm. in diameter), previously soaked in the antibiotic solutions, were placed on the surface of cooled, inoculated nutrient agar in petri dishes. Triplicates were run for each antibiotic on the inoculated nutrient agar at *p*H levels of 6.0, 6.5, and 7.0. For the control the discs were dipped in sterile water and used as above. Measurements of the resultant clear rings indicating inhibition were taken after 48, 72, 120, and 144 hours of incubation at 37° C. Bacitracin was selected because it gave the greatest sustained inhibition and was especially effective at *p*H 6.0. This is the optimum *p*H for growth of normal sunflower-callus tissue *in vitro*.

Activity of bacitracin in medium.—An experiment was run to determine the effectiveness of bacitracin on the contaminant in a medium used for the culture of sunflower-callus tissue. This medium contained salts, sugar, vitamins, amino acids, adenine sulfate, indoleacetic acid, and agar in proportions as described by Henderson *et al.* (*1*). The culture medium, in 10 cc. portions, was distributed in 4 groups of 5 tubes each. The final bacitracin concentrations for the 4 sets of media were 0, 10, 50, and 100 ppm, respectively. Needle stabs of *Bacillus* sp. from 18-hour plate cultures were made in the tubes which were then incubated at 25° C. for 8 weeks.

The organism continued to grow well after 2 weeks in the media containing 0 and 10 ppm bacitracin, but growth was lacking in the media containing 50 and 100 ppm bacitracin. At the end of 4 weeks, some growth was seen in the medium containing 50 ppm but none was observed in the medium containing bacitracin at 100 ppm. After 8 weeks of incubation, there was still no evidence of bacterial growth in bacitracin at 100 ppm. Thus, bacitracin at 100 ppm in the culture medium used for sunflower-callus tissue will inhibit the growth of the *Bacillus* sp.

The effect of bacitracin on the organism in the presence of the tissue.—Another experiment was conducted to test further the effectiveness of

[4] This is the classification given the organism by the public health laboratory to which the organism was sent for identification. The identification was not complete.

[5] Most antibiotics are heat labile, depending on the *p*H of the medium.

bacitracin on the growth of the organism under the conditions induced by actively growing sunflower-callus tissue. The effect of "aging" of bacitracin in the medium was also tested.

Four lots of medium containing bacitracin at 5, 10, 50, and 100 ppm, respectively, were prepared simultaneously. Each lot consisted of 20 tubes. Five tubes of each lot were inoculated immediately with contaminated tissues. During the next 3-week period, 5 additional tubes of each lot were inoculated weekly until the remaining 15 tubes were used. Weekly observations of bacterial growth in the medium around the tissues were made and recorded (table 1).

TABLE 1.—*Contaminated cultures treated with bacitracin (4 or 5 tubes per concentration)*

Bacitracin concentration (*ppm*)	Age of medium at time of inoculation with tissue	No. of tubes contaminated. Age (*in weeks*) of cultures								Total tubes contaminated
		1	2	3	4	5	6	7	8	
5	0	4	–	–	–	–	–	–	–	17/17
	1	1	2	–	1	–	–	–	–	
	2	3	–	–	1	–	–	–	–	
	3	3	2	–	–	–	–	–	–	
10	0	3	1	–	–	–	–	–	–	19/19
	1	5	–	–	–	–	–	–	–	
	2	5	–	–	–	–	–	–	–	
	3	1	2	–	–	–	–	2	–	
50	0	–	–	–	–	1	–	–	–	8/20
	1	–	–	–	2	1	–	–	1	
	2	–	1	1	–	–	–	–	–	
	3	1	–	–	–	–	–	–	–	
100	0	–	–	–	–	–	–	–	–	3/20
	1	–	–	–	1	–	–	–	1	
	2	–	–	–	1	–	–	–	–	
	3	–	–	–	–	–	–	–	–	

The criterion used to detect or indicate the presence of bacteria was the characteristic "halo" formation and fogging in the tissue area. Nearly all the bacterial growth, when present, occurred during the first 4 weeks after inoculation. Bacterial growth was profuse and contamination complete in the tubes containing 5 and 10 ppm bacitracin. Growth of the contaminating organism was inhibited 60 percent at the 50 ppm level and 85 percent at the 100 ppm level of bacitracin. The tissues that were free of visual contamination in the medium were firm in texture, while those which grew in the presence of contamination were soft. Age of the medium had no influence on the effectiveness of bacitracin to inhibit contamination.

Bacitracin as a growth stimulant.—In addition to the action of bacitracin upon the contaminating organism, its effect on the growth of sunflower-callus tissue itself was considered. Nickell (*2*) reports that bacitracin and 4 other antibiotics at 5 ppm stimulated the growth of virus tumor

tissue from *Rumex acetosa* and duckweed (*Lemna minor*). The latter also was stimulated considerably by penicillin G (*3*). De Ropp (*4*) found that both penicillin G and streptomycin at levels of 50 to 500 ppm caused an inhibition in growth of sunflower tumor tissue, whereas normal tissue thus treated resulted in an increased weight over controls. He concludes, however, that these results may be due to traces of indoleacetic acid in the penicillin preparations. Thus, one might obtain either accelerated or inhibited growth of tissues grown *in vitro*, depending on the type of tissue and plant used and the purity of the preparation.

In the case reported here bacitracin did not appear to act as in the case reported by Nickell above, at least not at 100 ppm. Using aseptic tissues at 100 ppm, there is an inhibition (10–25%) in growth over the 8-week period. This is probably caused by the bacitracin itself (table 2).

TABLE 2.—*Average wet weight of 20 normal sunflower tissues grown* in vitro *for 8 weeks on the standard medium with or without bacitracin at 100 ppm*

Medium	Expt. I	Expt. II	Expt. III
	(*mg.*)	(*mg.*)	(*mg.*)
With bacitracin	965	708	779
Without bacitracin	1,032	931	807

Summary

A bacterial contaminant isolated from infected sunflower tissues grown *in vitro* was subjected to various concentrations of penicillin, aureomycin, streptomycin, and bacitracin. Only bacitracin retained its antibiotic effect upon autoclaving the medium. Bacitracin was subsequently used at concentrations of 5, 10, 50, and 100 ppm against both the contaminating organism and the infected tissue. Only the level of 100 ppm was effective against the organism alone and in the infected tissue. It would appear that the level of 100 ppm is inhibitory to the growth of the sunflower tissue culture itself.

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A New, Inexpensive Circling Device for Marking Specific Areas on Tissue Smears or Sections [1]

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In the field of exfoliative cytology the need for relieving the pathologist of reviewing the bulk of the material examined is evident. Since properly trained technicians can reliably screen the slides, a uniform method of indicating questionable cells for further study by the pathologist is desirable.

One technique for indicating these cells is to place an ink dot to one side of the cell, or group of cells, in question. Because this system has many disadvantages, the device described here was developed to properly encircle the cells which required further study.[4]

The materials required to make the circling device are: a 1½-inch tip of a finger from a thin rubber (latex) glove, a wooden applicator stick 1 to 2 mm. in diameter, rubber cement, and a #62 printer's letter "O," procurable from any rubber-stamp dealer.

The convex center of the rubber-glove finger tip is stretched tightly over the end of the applicator stick, causing a slight projection. The tip end of the applicator stick and the rubber tip over it are cut off forming a hole which just fits the printer's letter "O." A drop of rubber cement is placed on the rubber base of the printer's "O" which is then balanced on the end of one's finger. The perforated glove tip is then pulled over the finger and the "O" is pushed through the opening in the rubber. The rubber base of the "O" is fixed to the glove tip by the rubber cement.

Allow a minute for the cement to dry. This device may now be pulled gently over the oil-immersion objective which is first removed from the microscope. The circle is then adjusted to the center of the lens. The wooden applicator may be useful for this purpose (fig. 1).

An ordinary stamp pad is lightly touched to the "O." The cell, or group of cells, to be indicated is located and placed in the exact center of the low-power field. The microscope nose is raised, the oil-immersion

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[2] Cytodiagnosis Service, Pathologic Anatomy Branch, National Cancer Institute, National Institutes of Health Public Health Service, U. S. Department of Health, Education, and Welfare.

[3] St. Luke's Hospital, Cleveland, Ohio.

[4] A similar device was listed in the Erb and Gray catalogue in 1949, but is no longer available because of expense involved. (Personal communication: Erb and Gray, 1955.) Our marking device was separately developed in 1949.

objective rotated into place, and then the nose is lowered until the rubber circle just touches the slide without pressure. The desired cells are now clearly encircled. The marked areas should be checked from time to time under the 16 mm. objective to be sure that the circle has not slipped and is properly centered.

The marker can be used four or five times before requiring a fresh application of ink. The ink application is sufficiently light that it does not obscure the cells beneath it and a continuous field can be examined. The rubber tip can be rolled off the oil-immersion objective at any time. It causes no damage to the lens.

After using this device regularly for several months two difficulties were noted: 1) The heat from the microscope lamp caused the tip of the rubber glove to deteriorate. 2) Since the circle is not immovably attached, care must be taken to avoid displacing it, and it will need occasional adjustment.

To make the device more permanent and stable we now use an old oil-immersion objective from which the outer lens, *i.e.*, the boot lens, is removed. The opening for this lens is slightly enlarged by forcing a six-penny carpenter's nail through the opening. The letter "O" is then affixed to the second or inner lens. The boot is replaced and the marker can now be used in the same fashion as in the originally described device (fig. 2).

The ink circles are easily located on the slide by the pathologist reviewing the case, and the slides can be filed with the circles still intact. The marking has remained clear through the 3-year period we have used the device.

This device is also of value to the pathologist, the hematologist, or the bacteriologist in locating fields for further observation or photography, since the size and shape of the marker can be varied.

Summary

A device for marking fields on microscopic slides is described. It has been found superior to any previously used method of marking.

Special advantages gained through using this device are: Cells for future observation or photography are delimited within a circle; a trained technician can locate them for the pathologist; and the marking remains clear so that the pathologist can identify and review the slides at his convenience.

The technique is simple, inexpensive, accurate, and time-saving.

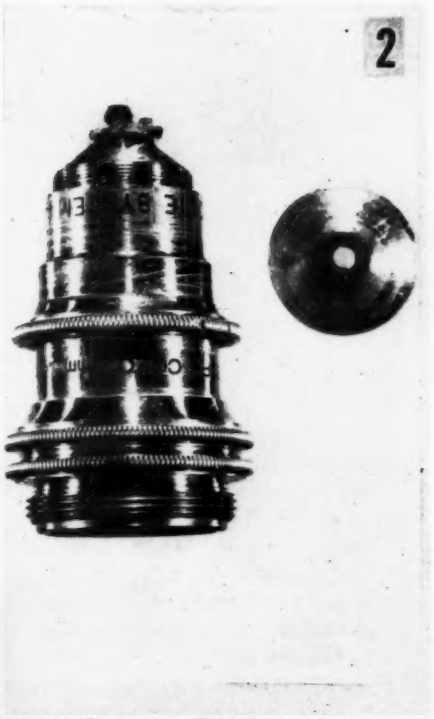
PLATE 85

FIGURE 1.—Microscope showing circling device on oil-immersion objective.

FIGURE 2.—Improved circling device with marker permanently mounted in oil-immersion objective without lens system.

FIGURE 3.—Slide showing the circles made by the device.

FIGURE 4.—Microscopic view of circle at low magnification showing group of cells which have been marked. × 55



366462—56——21

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366462—56——21

Preliminary Studies in a Quantitative Approach to Skin Carcinogenesis [1,2,3]

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The cutaneous application of materials to experimental animals is frequently employed to ascertain skin-tumor-inducing potency; yet studies on dose-response relationships for cutaneous activity, even of recognized experimental carcinogens, are conspicuously scarce in the literature. This report describes some preliminary results obtained in a dose-response study which utilized calibrated drop applications of a chemical carcinogen for a quantitative approach to skin-tumor induction. We sought to develop a practical method for quantifying the amount of test substance delivered to the skin of experimental animals and to determine whether some standards of carcinogenic activity might be achieved for evaluating comparative potency. The studies thus far have indicated that these objectives can be achieved, and dose-response relationships for skin carcinogenicity can be established in terms of biologic response to a chemical carcinogen, such as 3,4-benzpyrene, delivered to a localized area in microgram quantities.

Materials and Method

Strain C57BL/6 male mice, 8 to 17 weeks of age on initial exposure, were shaved periodically in the interscapular and adjacent thoracic region with electric clippers. Test solutions were applied to that area dropwise. Inexpensive droppers were made by filing off the points of hypodermic needles, smoothing the outer and inner surfaces of the blunted tips, and mounting them on 1 cc. tuberculin syringes. The gauge of hypodermic needle used was determined by the size of drop desired; for example, drops of 0.008 cc. toluene (127 $\pm$ 2.4 drops per cc.) were obtained consistently with a 20-gauge needle (table 1). Excellent control to secure dropwise

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[3] Presented in part at the annual meeting of the American Association for Cancer Research, Atlantic City, N. J., 1954.

[4] The authors take this opportunity to thank Mary C. Stezoski, Raymond Wright, and Seena Lewine for devoted technical assistance in these studies. For statistical analysis and table 1, we are grateful to Zdenek Hrubec, graduate student, Department of Biostatistics, Graduate School of Public Health, University of Pittsburgh.

TABLE 1.—*Drop delivery of toluene with blunted hypodermic needles*

Needle gauge	Drops per ml.* (*mean*)	Coefficient of variation (*percent*)
15	70. 7	3. 4
16	73. 3	3. 2
17	82. 0	2. 9
18	94. 6	2. 5
20	127. 3	1. 9

*Mean number of drops per milliliter is based on 18 determinations per needle size. These consisted of three 1 ml. determinations on two needles of the same gauge by each of three different operators.

$$\text{The coefficient of variation} = \frac{\text{standard deviation}}{\text{mean}} \times 100$$

A standard deviation of 2.4 drops per ml. was estimated from the pooled performance of the operators after variations due to needle size, variation between two needles for each size, and variations due to operators were isolated.

delivery was possible by rotating the syringe plunger while depressing it gently until a drop fell.

Standard carcinogenic solutions consisted of commercially available 3,4-benzpyrene [5] dissolved in benzene [6] in weight per volume concentrations of 0.01 to 1.0 percent. Individual drops of the benzpyrene solutions were applied to the interscapular skin of groups of mice thrice weekly. The estimated amounts of carcinogen so deposited were made to vary from 1 to 100 μg. per application. Control mice received drop applications of benzene in the same region. Treatments were continued until the mouse developed a persistent tumor of the exposed skin or died of other causes. The term "tumor" as used in this report refers to papillomas and epitheliomas on the interscapular or adjacent skin which persisted for at least 2 weeks and attained a diameter of at least 5 mm.

Results

The consistency of drop size obtained with this method of application is shown in table 1. Assuming that the volume of each drop is not affected significantly by the amount of benzpyrene in solution within the concentration range of 0.001 to 1.5 percent,[7] it can be estimated, for example, that with a 20-gauge needle delivering 127 drops per cc., the amount of benzpyrene per drop can be made to vary from 0.08 to 120 μg.

Dose-response relations elicited with this method on C57BL/6 mice are shown in table 2.

A comparison of the intervals of chronic exposure by this method of application indicates that for the different experimental groups within the estimated dose range of 1 to 50 μg. per application, the duration of time preceding tumor appearance is an inverse function of the concentration of carcinogen per drop.

[5] Hoffmann-LaRoche Inc., Nutley, N. J.

[6] Toluene is currently used instead of benzene as our standard solvent because of its lower volatility.

[7] The validity of this assumption has not actually been tested because it is believed that any changes in drop size due to benzpyrene in the designated concentration would be insignificant.

TABLE 2.—*Time of appearance of tumor and range in biologic susceptibility of strain C57BL/6 male mice* treated with graded doses of 3,4-benzpyrene†*

Percent benzpyrene in benzene	Estimated dose per application (*μg.*)	Number of mice effectively exposed‡	Number of tumors in relation to time of appearance (*weeks*)															
			10 14	15 19	20 24	25 29	30 34	35 39	40 44	45 49	50 54	55 59	60 64	65 69	70 74	75 79	80 84	85 89	90
1	100	7	1	4	2													
0. 5	50	11	6	4	1													
0. 1	10	13		1	5	7												
0. 05	5	33		1	11	13	7	—	1									
0. 01	1	12				1	2	—	—	—	1	—	1	2	1	—	1	3
0. 01	1	30				1	—	3	4	—	1	2	1	§		§		§
None	1 drop of benzene	46																

*One to 18 weeks of age at initial exposure.
†Applied to shaved interscapular skin dropwise three times per week.
‡Number that lived at least 40 weeks after treatment began or developed tumors prior to that time.
§Survivors sacrificed because of a viral infection.

In any group of mice subjected to this type of dosing there is a spread in the time intervals at which response occurs. The duration of this spread is again an inverse function of the concentration of carcinogen per drop applied. This difference in susceptibility among the individuals of a group must be due, in part at least, to biologic factors. The spread in response time was greatest when the lowest dose was used—thrice weekly applications of 1 μg. of benzpyrene—and conversely, the spread was shortest when the highest dose was used—thrice weekly applications of 50 to 100 μg. of benzpyrene.

As an incidental observation, it is to be noted that at the estimated dosage level of 1 μg. of benzpyrene all effectively treated mice responded with tumors. Therefore, the threshold level for tumor induction by this substance must be of some value lower than the dose used here.

Discussion

The use of commercial 3,4-benzpyrene without repurification in this study introduced a possible error which, though uniform percentagewise for all concentrations employed, may have decreased the amount of carcinogen per drop by some small fraction of the amount calculated. Photochemical reactivity is another factor which may have reduced the potency of the benzpyrene solutions. On the other hand, the volatility of benzene tends to increase the concentration of carcinogen in solution, even if only when the drop is formed on the tip of the blunted needle. These factors are examples of remediable limitations of the approach used, the correction of which can increase its value as a more precise quantifying technique. Relative values developed with the technique as applied in the study are not affected by these considerations.

Estimates of quantitative applications of a carcinogen with the brush-painting technique were reported by Mider and Morton (*1*) and Cramer and Stowell (*2*), while previous reference to the use of measured drops as a way of controlling dosage may be found in the work of Maisin and Liégeois (*3*) and Cowdry and Suntzeff (*4*). The latter technique, in our experience, has been the superior one for periodic, consistent applications of minute amounts in small volume to many animals.

In published methods for grading activity of percutaneously applied chemicals, dosage has not been adequately considered as a determinant of carcinogenic potency. The two criteria heretofore suggested were tumor incidence and latent period (time interval of response) after liberal but unmeasured applications of an active agent (*5*, *6*). Possible explanations for the neglect of dosage as a criterion, inferred from the literature, may be related to the fact that 0.3 percent of a polycyclic agent in benzene has been reported as a "low standard concentration of carcinogen" (*7*), to the usual practice and technical limitations of applying test solutions to the skin of experimental animals by painting with a small brush, or to the observations that variations in concentration of the carcinogen within the limits of 0.3 and 1.0 percent have relatively little effect on the neo-

plastic responses of the skin (*2, 6–9*). Reports in the literature of no significant decrease in tumor-induction time with solutions more concentrated than 0.3 percent have been confirmed in part in this laboratory (table 2, reactions to 1.0 and 0.5% concentrations).

These latter observations support the impression that an optimal, or saturation, dose range exists for repeated carcinogenic stimuli, within which range the experimental animal develops a maximal carcinogenic response. The optimal level may be considered as the one characterized by the shortest duration of time of tumor appearance and the shortest spread of response times among experimental groups receiving different doses of the same agent. The lack of any significant decrease in time of tumor appearance reported in the literature for polycyclic carcinogens in the range 0.3 to 2.0 percent may therefore denote that the 0.3 percent level, rather than being a low standard concentration, is, in fact, within the optimal dose range for carcinogenesis.

The data in table 2 suggest that the potency of a carcinogen for the skin depends on dosage and can be varied according to dosage, provided the amounts involved do not exceed an optimal, or saturation, level. A similar inference for carcinogens injected subcutaneously may be drawn from the data of Bryan and Shimkin (*10*).

The concept that dosage at subsaturation levels determines the carcinogenic potency of an agent is in no way at variance with the findings of Cramer and Stowell (*2*), who reported that the period of induction is not much delayed for early tumors produced by a small total dose of 20-methylcholanthrene as compared with the carcinogenic effect induced by the use of large doses. In their study of the effect of dosage attenuation on skin-tumor induction, they fixed the amount of carcinogen per application and varied the time interval between successive applications. The minimal dose of 20-methylcholanthrene applied repeatedly in their study was 100 μg., while the maximum dose of our somewhat less potent agent, with which an increase in carcinogenic response was obtained, was 50 μg.

The potency of 3,4-benzpyrene at concentrations of 0.01 percent and in amounts of 1 μg. or less per repeated exposure has been demonstrated with DBA/2, CAF_1 (*11*), C57L, as well as with C57BL/6 mice. The high tumor incidence obtained at all dilution levels down to 0.01 percent benzpyrene shows how differences in agent activity are demonstrated by time of tumor appearance when animals are treated with small but adequate doses and are observed throughout their normal life span rather than for arbitrarily designated shorter periods. In our studies involving the detection of carcinogenic materials at low concentration, the estimation of comparative potency of carcinogens, and of biologic susceptibility to tumor induction, these preliminary results have demonstrated the advantages of exposing animals in a quantitative fashion to a range of dilutions of a standard carcinogen rather than to a single high concentration. This method of study requires utilization of the entire life span of the experimental animal, resulting in an increased cost which is justified by the more precise information produced.

Summary

1) A simple, quantitative approach to the study of dose-response relations in skin carcinogenesis is described.

2) Studies with this method have indicated that time of tumor appearance and differences in susceptibility to tumor induction among individual mice of a group are inverse functions of the microgram quantities of carcinogen applied. Conversely, carcinogenic response appears to be a direct function of dosage when mice are treated periodically with 1 to 50 μg. of benzpyrene per application.

3) The high incidence of skin tumors induced with doses as low as 1 μg. of benzpyrene per exposure indicates the need for more adequate awareness of carcinogenic potency at low concentrations.

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The Metabolism of Animal Tissues Cultivated *in Vitro*. I. Amino Acid Metabolism of Chick Embryonic-Heart Fibroblasts Cultivated in Synthetic Medium M 150[1]

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Nutritive media employed for the propagation of animal cells in tissue culture have in the past consisted almost exclusively of natural materials, such as blood plasma, serum, body fluids and exudates, and extracts of various tissues and organs. These natural materials have made it possible to cultivate a wide variety of tissue cells outside the body for prolonged periods of time, but because of the complex and undefined nature of these substances it has not been possible to determine precisely which components are actually utilized by the cells for survival and multiplication. The development of a chemically defined medium (*1*) that supports the survival of freshly explanted chick embryonic tissues for reasonable lengths of time has made it possible to study the effects of known variations in the medium on tissue-cell survival, and, at the same time, to determine any alterations in the medium resulting from cell growth and metabolism. It is the purpose of the present communication to report the first of a series of studies made in this laboratory on the utilization of amino acids by various freshly explanted tissues cultivated in synthetic medium M 150 (*2,3*).

Materials and Methods

Tissue-culture procedures.—All cultures employed in this study were prepared from the heart muscle of 11-day-old chick embryos. The tissue from a pooled lot of 10 to 16 hearts was chopped to a fine pulp with curved scissors and portions were transferred to the culture vessels by a capillary pipette. After a period of 25 to 30 minutes, during which the tissue became fixed directly to the glass without the use of plasma clots, medium M 150 was added and the cultures were incubated at 37° C.

In initial experiments, cultures containing from 1 to 2 mg. of tissue, wet weight, were prepared in standard pyrex test tubes and rotated by the conventional roller-tube technique (*4*). However, chromatographic analysis of the fluids from such cultures showed only small changes in total amino acids, and, accordingly, cultures containing 10 to 20 mg. of

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[2] The technical assistance of Miss J. Gagnon, Miss D. Slessor, and Mr. L. F. Guerin is gratefully acknowledged

tissue, wet weight, were prepared in T–30 flasks (*5*). The increased mass of tissue in relation to the volume of medium (3.0 ml.) resulted in satisfactory changes in total amino acids, and this type of culture was used in all subsequent experiments.

The synthetic medium used throughout this study was M 150 (*3*), which contains the organic ingredients of medium 199 (*1*) but is prepared in Hanks' balanced salt solution (*6*). After a preliminary incubation period of 5 to 7 days without change of M 150, the culture fluids were removed and replaced at least once a week for the duration of the experiments. Adjustment of the *p*H by gas mixtures rich in carbon dioxide was not found necessary.

Chromatographic techniques.—Changes in the amino acid composition of M 150 brought about by the living cells were measured by paper chromatography. Fluids from groups of 6 to 8 cultures prepared from the same tissue brei were pooled and 5.0 ml. samples used for analysis. These were concentrated to dryness *in vacuo* over H_2SO_4 and reconstituted in 0.2 ml. of deionized water. Five to 10 λ quantities of this concentrated material were found sufficient for satisfactory analyses, with one-dimensional descending paper chromatograms.

The solvent system used in most of these studies was a modification of Woiwod's *n*-butanol-acetic acid-water mixture (*7*). It was found that the proportions originally used by Woiwod for bacteriological media could not be applied to M 150, since no separation was obtained below the glycine and serine region. Consequently, extensive studies were made with modifications of Woiwod's solvents until a satisfactory mixture was obtained. The mixture finally adopted was prepared as follows: 55 ml. of *n*-butanol was saturated by shaking with water in a separatory funnel, the water layer removed, and 15 ml. of concentrated acetic acid added to the water-saturated *n*-butanol. This solvent mixture was found to provide good separation of most of the amino acids present in M 150.

In addition to this solvent system, the use of ethanol-water mixtures (80 to 85% ethanol in water), and of a mixture composed of 25 ml. of *n*-butanol, 25 ml. of 95 percent ethanol, and 5 ml. of deionized water was found helpful in increasing the degree of resolution of certain areas on the paper chromatograms. Various combinations of phenol and water were also tested but were found to be ineffective in separating the amino acids present in M 150. By using combinations of these various solvent systems on duplicate chromatograms, it was found possible to obtain a complete picture of the amino acids in the synthetic medium without the use of desalting procedures.

Schleicher and Schuell No. 597 paper was found most suitable for use with the butanol-acetic acid-water solvent system, and Whatman No. 1 for use with alcoholic solvents. All chromatograms were routinely developed at room temperature for 16 to 18 hours and were then dried at 110° C. for 2 to 5 minutes. Ninhydrin-positive material was detected by spraying with 0.2 to 0.4 percent ninhydrin dissolved in either water-saturated *n*-butanol or 85 percent ethanol. The chromatograms were

allowed to dry for 5 to 10 minutes at room temperature and were then heated to 110° C. for 3 minutes. The chromatograms were read, stored in the dark for 24 hours to permit any further changes in color intensity, and read a second time.

It was observed that separation and resolution could be increased by carefully drying the chromatograms at 110° C. until excess solvent was removed (2 or 3 minutes) and then redeveloping for a second 18-hour period in the same solvent system before applying ninhydrin. Drying the chromatograms between the two development periods was found to yield a more effective separation of the amino acids than could be obtained by one continuous 36-hour development. The illustrations shown (figs. 1, 2, and 3) were obtained by this two-step procedure.

In addition to the general ninhydrin procedure, proline was determined separately by the isatin method (*8*) and total α-keto acids by chromatography as 2,4-dinitrophenylhydrazones (*9*). Each of the amino acids present in M 150 was dissolved in Hanks' solution and run individually on paper chromatograms with the various solvent systems employed in order to establish its relative position on the chromatograms under these experimental conditions. All chemicals used in the preparation of solvent systems and developing solutions were of reagent grade.

Experimental Procedures and Results

Amino acid changes during early cultivation periods.—At frequent intervals during the cultivation of freshly explanted chick embryonic-heart tissues in synthetic medium M 150, the culture medium was removed, concentrated, and analyzed on paper chromatograms for changes in amino acid composition. Samples from each lot of M 150 used on the cultures were concentrated to dryness at the time of preparation and included on the chromatograms as controls. The results of a typical experiment, carried out over a 3-week period, are shown in figure 1. The culture fluid removed after the first 7 days' cultivation shows very little change from the control medium, other than a possible decrease in glutamic acid and a marked increase in alanine. However, the fluid removed from the cultures on the 19th day of cultivation (after 7 days' exposure to the tissues) shows marked changes from the control medium. Cystine, lysine, histidine, and arginine, present in the lower region of the chromatogram, appear to have been completely removed, while some decrease is also apparent in the leucine and isoleucine area at the top of the chromatogram. In addition to these changes, increases are to be seen in the alanine, methionine, and valine regions. Quantitative chemical studies (to be published) have shown that approximately 85 percent of the arginine originally present has been removed from the medium.

Amino acid changes during prolonged cultivation.—In view of the metabolic activity shown by the tissues after 19 days' cultivation, the experiment was continued to 36 days, at which time the cultures had begun to degenerate and die. A chromatographic analysis of the fluids removed

from these cultures at various time intervals is presented in figure 2 The most striking changes have occurred in the basic amino acid region at the bottom of the chromatogram. Fluids removed at 21 and 25 days (Nos. 6 and 8), after 2 and 4 days' exposure to the tissues, respectively, show almost complete uptake of cystine, lysine, histidine, and arginine. As the cultures age, however, this amino acid utilization decreases, and by the 36th day relatively little change can be seen. The increase in ninhydrin-positive material in the methionine region, which was quite pronounced during the early culture stages, is still shown at the 21st day, but is not apparent beyond that time.

Amino acid changes during tissue cultivation shown on paper chromatograms developed with alcoholic solvents.—The butanol-acetic acid-water solvent system (figs. 1 and 2) afforded good resolution of the amino acids of M 150, particularly in the region of the basic amino acids. To complete the picture of amino acid changes which occurred in the synthetic medium during tissue cultivation, repeated experiments were carried out with alcoholic solvent systems which give a different pattern of separation of the amino acids on chromatograms. The results of a typical experiment, in which the ethanol-butanol-water development system was employed, are presented in figure 3. In this solvent system, the basic amino acids have not separated from glutamic and aspartic acids, but good separation has been obtained in the intermediate regions containing serine, glycine, threonine, alanine, proline, and hydroxyproline. With this solvent system, a considerable uptake of tryptophan is apparent and the increases in the serine, glycine, threonine, alanine, and methionine regions, observed in the previous two chromatograms, are confirmed. As in the case of the previous chromatograms (figs. 1 and 2), the amino acid changes in the medium are found to be most apparent during the early and intermediate stages of tissue cultivation and become practically negligible once the cultures reach the stage of degeneration.

Effect of age of chick embryonic-heart cultures on amino acid changes in medium M 150.—The amino acid changes portrayed in figures 1, 2, and 3, and the results of a series of additional experiments, have been grouped according to the age of the cultures. These results are summarized in table 1. It is evident that chick embryonic-heart tissues actively remove cystine and the basic amino acids lysine, histidine, and arginine from medium M 150 and also utilize valine, phenylalanine, tryptophan, isoleucine, and leucine to a considerable extent. While these amino acids have been removed from the medium, the glutamine, serine, glycine, threonine, alanine, and methionine regions have increased in intensity. The changes observed with aspartic acid, hydroxyproline, and tyrosine were slight, and further studies are necessary to assess their significance. Variable results, including both marked uptake and marked release, were obtained at different times with glutamic acid. From all these results, it must be concluded that the amino acid metabolism of the tissues is influenced markedly by the length of the cultivation period.

Throughout these studies. the extent of the tissue metabolism of

TABLE 1.—*Changes in amino acid composition of synthetic medium M 150 during cultivation of chick embryonic-heart tissue* [1]

Amino acid	Age of culture in days [2]					
	7	19	21	25	28	36
Cystine	*	***	***	**	**	*
Lysine	0	**	**	**	0	0
Histidine	0	**	**	**	0	0
Arginine	0	***	***	**	**	*
Glutamine	+++	+++	0	0	+	+
Aspartic acid	0	*	*	0	0	0
Serine	++	++	0	0	+	+
Hydroxyproline	0	*	*	*	*	*
Glycine	++	++	0	0	+	+
Glutamic acid	**	++	*	*	0	0
Threonine	++	+	0	0	0	0
Alanine	+++	+++	*	0	+	+
Proline	+	+	+	+	0	+
Tyrosine	+	+	+	+	+	0
Tryptophan	0	**	**	**	*	*
Methionine	++	+++	+++	++	0	*
Valine	0	0	**	**	*	*
Phenylalanine	*	*	**	**	*	*
Isoleucine	0	*	**	*	*	*
Leucine	0	*	**	*	*	*

[1] All changes listed represent alterations from unused M 150 controls.
[2] +=Slight increase, ++=moderate increase, +++=marked increase.
*=Slight decrease, **=moderate decrease, ***=marked decrease.
0=No change.

tryptophan was difficult to determine because of the low concentration of this amino acid in M 150 (*1,3*) and because of the low intensity of the yellow color formed with ninhydrin (*10*). However, it was found that after the developed chromatograms had aged for 1 to 2 weeks, the yellow color of the tryptophan-ninhydrin reaction product was more definite, and changes in the intensity could then be detected with relative ease, even though this color does not show up well on photographic reproduction (figs. 1, 2, and 3). Somewhat similar difficulties were encountered with proline and hydroxyproline, but these difficulties were largely overcome by the application of the isatin method (*8*).

Discussion

Up to the present time, the use of synthetic media in the study of tissue cultures has been restricted to the effect of variations in the medium on cell multiplication (*11*) or tissue cell survival (*2, 3*), and little attention has been paid to the effect exerted by the cells upon the chemical composition of the medium itself. The present studies, using an incomplete medium, have shown that marked changes occur in the amino acid content of synthetic medium M 150 during the cultivation of fresh chick embryonic-heart explants. The most striking alteration in the composition of the medium is the rapid and almost complete removal of the basic amino acids lysine, histidine, and arginine, and further investigations on the tissue requirement for these amino acids are in progress.

The observation that cystine is completely removed from the medium

while the methionine region increases in concentration is in good agreement with the previous demonstration (*2*) that cystine, but not methionine, is essential for the survival of chick embryonic-heart tissue. It should be pointed out that the increase in the methionine region cannot be attributed definitely to methionine at the present time, since other uncharacterized ninhydrin-positive substances may have been elaborated by the cells. However, the location of this spot in the exact methionine region on chromatograms developed both by butanol-acetic acid-water and by alcoholic solvents suggests strongly that it is either methionine or a closely related breakdown product of methionine. In this connection, also, it was observed that the increase in the methionine region seemed to be proportional to the uptake of the basic amino acids as well as to the uptake of cystine. Thus, the increase in the methionine region was less marked in the later cultural stages when the basic amino acid uptake was relatively slight, even though cystine was still being removed from the medium to an appreciable extent. The significance of this observation remains to be determined.

The observation that certain amino acids are increased in the medium may conceivably be related to the enzymatic activities of the cells, quite apart from their utilization of amino acids for protein synthesis and growth. Thus, glutamine increases very considerably during the early cultural stages, while the glutamic acid content of the used culture fluids fluctuates. Since M 150 contains adenosinetriphosphate (ATP) at an appreciable level, it is possible that amidation of glutamic acid to glutamine may have occurred through the mediation of an ATP-dependent enzyme system (*12*). Similarly, the marked increase in alanine concentration suggests that transamination reactions may be proceeding vigorously, and determination of total α-keto acids by the nitrophenylhydrazone method (*9*) showed that these compounds were accumulating in the culture fluids. This accumulation of keto acids during cultivation of chick embryonic-heart fibroblasts in M 150 is interesting, in view of the report by Westfall, Peppers, and Earle (*13*) that keto acids are formed in large quantities during the cultivation of strain HeLa cells in protein-rich media.

The results of the present investigation have shown that marked decreases occurred in the concentration of certain of the amino acids in M 150 during tissue cultivation. These decreases could conceivably have resulted either from uptake of amino acids by the cells or from destruction of amino acids in the medium through enzymatic activity of the cells. Quantitative chemical studies (to be published) have shown that, while arginine is almost completely removed from the medium, the end-products of arginine metabolism (citrulline and ornithine) cannot be detected in the medium. From this observation, it must be concluded that the arginine disappearance represents an actual uptake by the cells and not a simple enzymatic breakdown. It has also been established that extensive degeneration and autolysis of the cultures is not accompanied by the release of free amino acids into the surrounding medium. It would appear, therefore, that the amino acids taken up by the cells are not

retained in a free form but are bound into either peptides or protein, as had been shown earlier by Gerarde, Jones, and Winnick (*14*) with embryonic heart and lung tissues cultivated in a protein-rich medium. Based on these considerations, the use of the word "metabolism" to describe the observed changes was considered justified.

From the results summarized in table 1, it is evident that the metabolic activity of the tissues toward amino acids changes with age of the cultures and that distinctly different patterns of activity can be detected at different time periods when the tissues are cultivated in medium M 150. Thus, three different phases of amino acid metabolism may be recognized: 1) During the first week of cultivation, while small amounts of embryonic-tissue juices would be adhering to the tissues and released into the medium from damaged cells, the uptake of amino acids is relatively slight, except in the case of glutamic acid. However, during this period, considerable increases in the glutamine, serine, glycine, threonine, and alanine regions were detected. 2) At about the third week of cultivation, when fresh chick embryonic-heart cultures in M 150 ordinarily show their greatest extent of proliferation on microscopic examination (*3*), the metabolism of amino acids appears to be at its maximum. This is shown by the almost complete removal from the medium of cystine and the basic amino acids lysine, histidine, and arginine. At the same time, considerable removal of valine, phenylalanine, tryptophan, isoleucine, and leucine takes place. This period of optimum activity appeared to persist until about the 25th day, with little release of amino acids into the medium throughout this time. 3) During the period from the 25th to the 36th day, while the cultures were still alive, nearly half the cells showed signs of degeneration, as determined by microscopic examination, and a considerable reduction in cell numbers had occurred due to cell death and autolysis. During this period, amino acid changes in the medium were almost negligible, and it was concluded that cell degeneration and death were not accompanied by a marked outpouring of amino acids into the surrounding medium.

In the present investigation, the use of a completely synthetic mixture has made it possible to study amino acid metabolism in the complete absence of the complex interrelationships that occur when protein is present in the medium. The changes that occurred in the amino acid content of the synthetic medium under these conditions agreed closely with the changes observed by Westfall, Peppers, and Earle (*13*) during cultivation of strain HeLa cells in a complex natural medium. It has been reported, however, that during cultivation of strain HeLa cells (*13*) or chick embryonic-heart tissues (*15,16*) in protein-rich media, the greater part of the cellular protein is derived not from the free amino acids and peptides but from the soluble protein of the medium.

Summary

Paper-chromatographic techniques have been developed that permit the separation and resolution of the amino acids present in synthetic medium M 150, without the use of desalting techniques.

Fresh chick embryonic-heart explants cultivated in medium M 150 have been shown to remove cystine and the basic amino acids lysine, histidine, and arginine quantitatively from the medium. Marked uptakes of valine, phenylalanine, tryptophan, isoleucine, and leucine were also observed.

During tissue growth in M 150, increases in the glutamine, serine, glycine, threonine, alanine, and methionine regions were detected in the medium. Little or no change was found with aspartic acid, hydroxyproline, proline, and tyrosine.

Different patterns of amino acid metabolism have been shown to occur, depending upon the length of the tissue cultivation period. With freshly explanted chick embryonic-heart tissues, maximal activity was found to occur during the period from the 14th to 25th day of cultivation.

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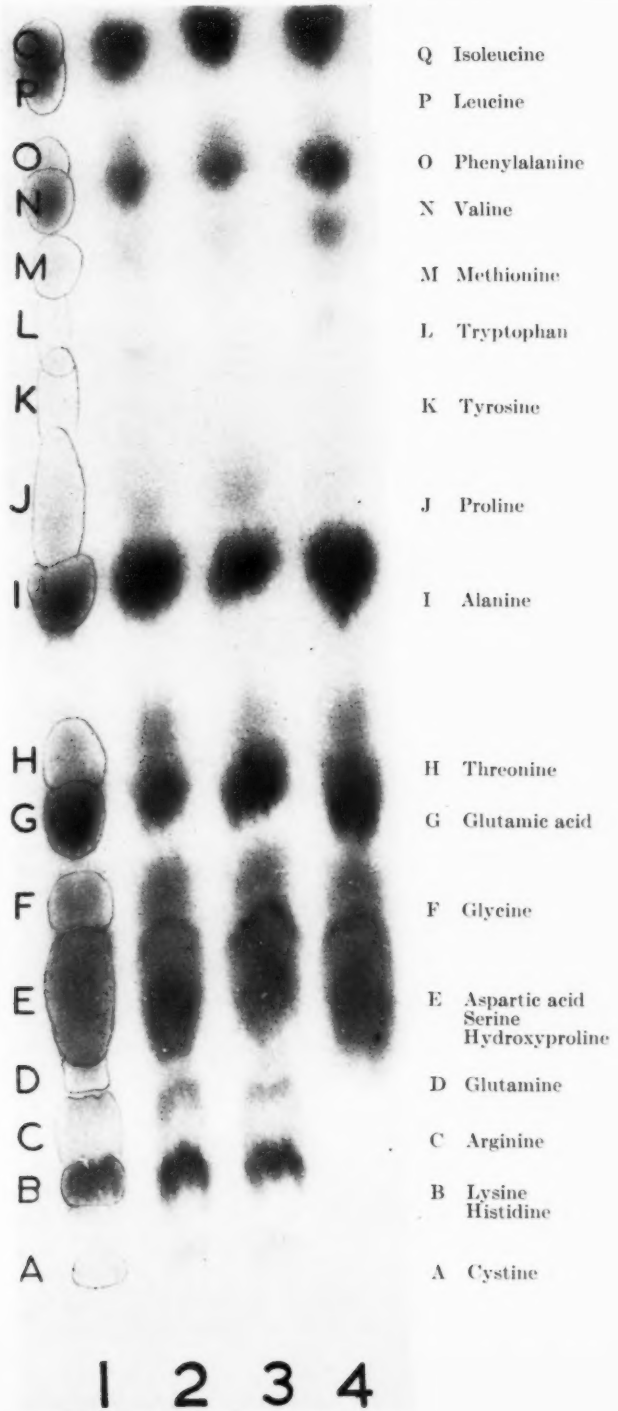
PLATE 86

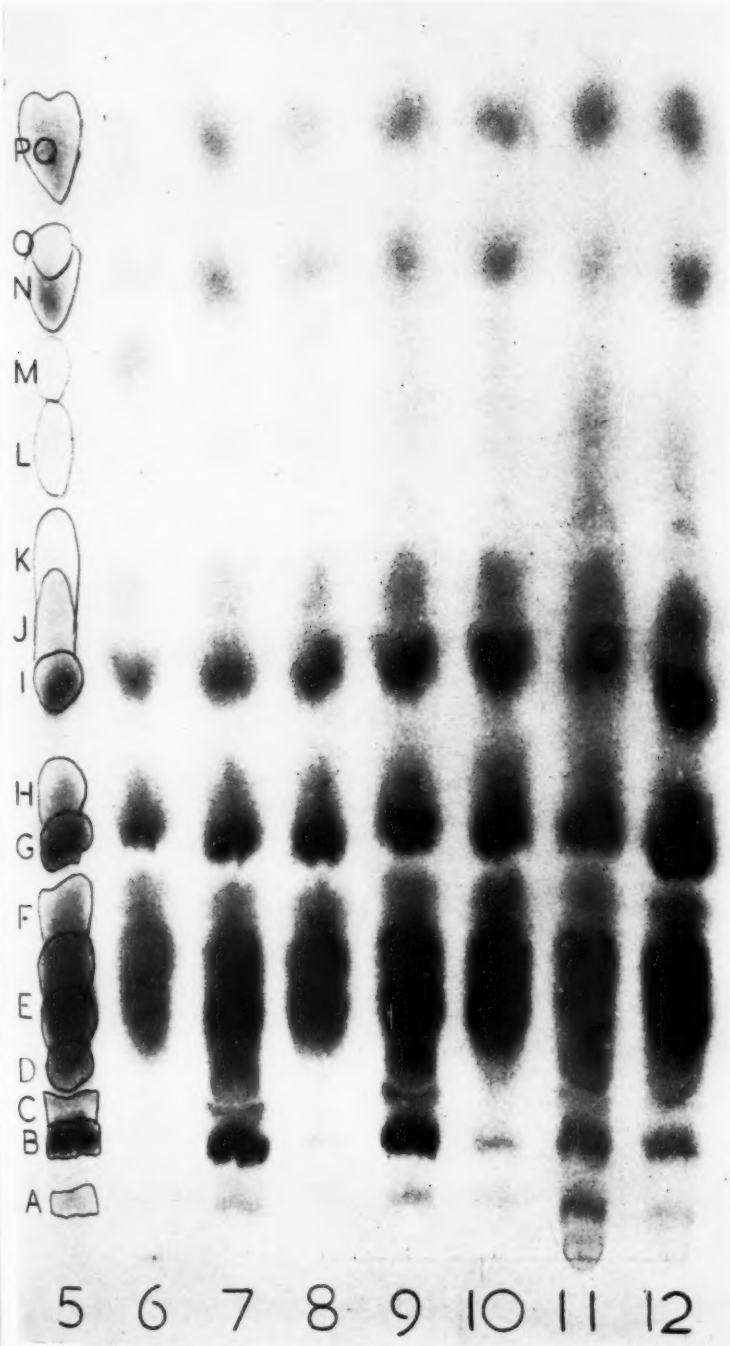
FIGURE 1.—Amino acid changes in synthetic medium M 150 during early cultivation periods of freshly explanted chick embryonic-heart tissues. Chromatogram developed with butanol-acetic acid-water mixture. Nos. 1 and 3=M 150 controls. No. 2=M 150 removed from cultures after first 7 days' cultivation. No. 4=M 150 removed from cultures on 19th day after 7 days' exposure to the tissues.


Q Isoleucine
P Leucine
O Phenylalanine
N Valine
M Methionine
L Tryptophan
K Tyrosine
J Proline
I Alanine
H Threonine
G Glutamic acid
F Glycine
E Aspartic acid
Serine
Hydroxyproline
D Glutamine
C Arginine
B Lysine
Histidine
A Cystine
1 2 3 4

366462—56——23

PLATE 87

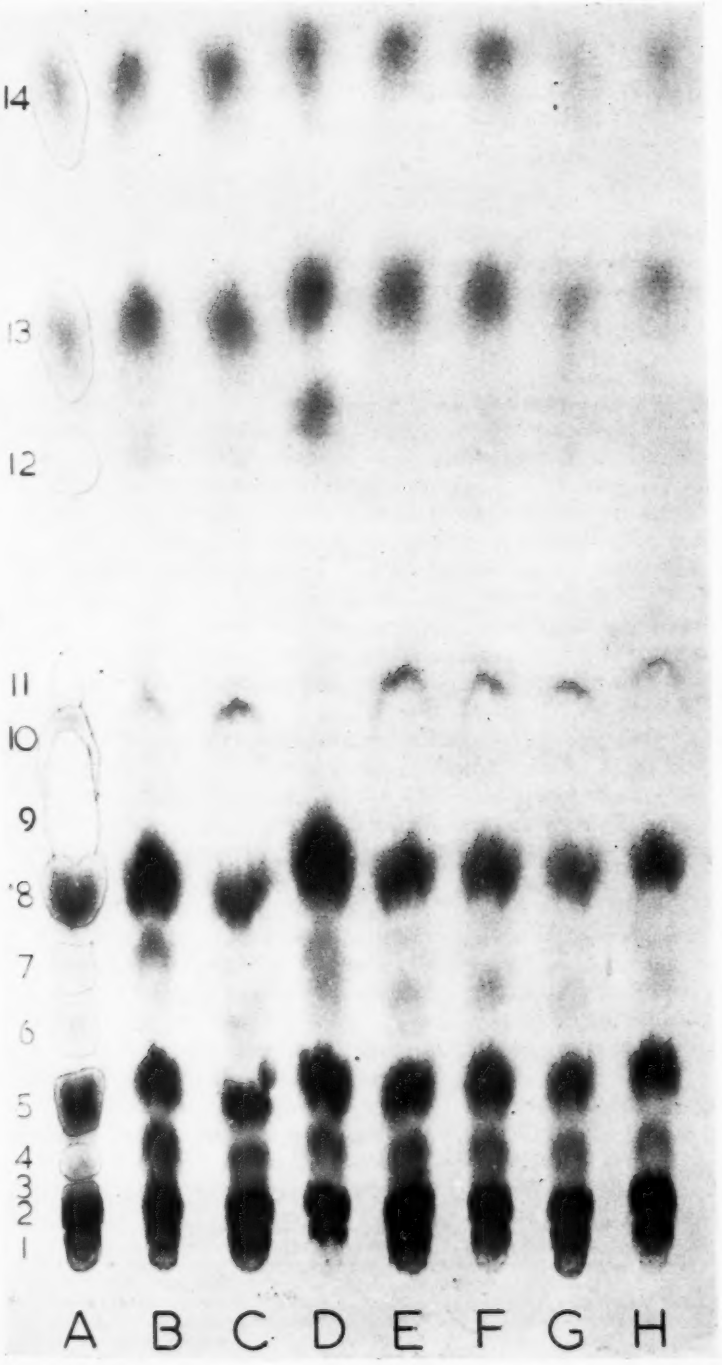
FIGURE 2.—Amino acid changes in synthetic medium M 150 during late cultivation periods of freshly explanted chick embryonic-heart tissues. Chromatogram developed with butanol-acetic acid-water. Nos. 5, 7, 9, and 11 = M 150 controls. No. 6 = culture fluid removed on 21st day, after 2 days' exposure to the tissues. No. 8 = fluid removed on 25th day, after 4 days' exposure to the tissues. No. 10 = fluid removed on 28th day, after 3 days' exposure to the tissues. No. 12 = fluid removed on 36th day, after 8 days' exposure to the tissues.



Pasieka, Morton, and Morgan Figure 2 1007

PLATE 88

FIGURE 3.—Amino acid changes in synthetic medium M 150 during cultivation of freshly explanted chick embryonic-heart tissues. Chromatogram developed with alcoholic solvent mixture. A, C, E, and G = M 150 controls. B = fluid removed from cultures after first 7 days' cultivation. D = fluid removed on 19th day, after 7 days' exposure to the tissues. F = fluid removed on 28th day, after 3 days' exposure to the tissues. H = fluid removed on 36th day, after 8 days' exposure to the tissues.



Pasieka, Morton, and Morgan Figure 3 1009

366462—56——24

Effects of Some Metabolic Inhibitors on the Growth and Lipide Composition of Earle's Strain L Cells[1,2,3]

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Although a number of respiratory inhibitors have been studied in connection with plant root, yeast and bacterial metabolism, or with tissue slices and homogenates, relatively few studies have dealt with their effects on cells growing in tissue culture. Pomerat and Willmer (*1*) studied the effects of sodium azide on the mitotic index and the cytochrome oxidase of chick periosteal fibroblasts. Verne (*2*) reviewed some of the work on dinitrophenol in relation to its toxicity on chick-heart fibroblasts or nervous tissue. More recent studies by Osgood and Chu (*3*) have dealt with the effects of nitrogen mustard on granulocytic cells, while the latest reports by Danes and her collaborators (*4*, *5*) show the effects of cyanide on the respiration and growth of 7-day chick-heart fibroblasts in tissue culture.

In conjunction with some studies on the influence of metabolic inhibitors on the respiration of Earle's strain L mouse cells (*6*, *7*) we have investigated the effects of 2,4-dinitrophenol (DNP), iodoacetic acid (IAA), and sodium azide (NaN_3) on the multiplication of these L cells cultivated on glass.

Materials and Methods

Cultures of L cells were maintained as previously described (*8*). Stock cultures were grown in T-60 flasks in our standard medium consisting of 40 percent horse serum, 40 percent Morgan, Morton, and Parker's mixture 199 (*9*), and 20 percent Tyrode's solution. The inoculum used was approximately the same in all tests; the optimum amount was found to be about 200,000 strain L cells per D-3.5 Carrel flask.

Cells from an actively growing stock culture were washed with 0.25 percent trypsin in Tyrode's solution to provide the initial inoculum. Trypsin was used, as it was found to be effective in removing the cells from the glass, in dissolving extraneous protein material and nonliving

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cells, and in facilitating the dispersion of the strain L cells for counting. The strain L cells remained in contact with the trypsin solution for a half hour at 37° C. The cell suspension was then pipetted into a 15 ml. centrifuge tube and centrifuged at low speed for about 5 minutes. Fine cellular debris or precipitate remained suspended, while the L cells were packed at the bottom of the tube. The supernatant fluid was decanted and the cells resuspended in Tyrode's solution, or the medium to be tested, and diluted to the desired concentration.

Duplicate counts were made in a hemocytometer of the cells in the trypsin solution. The cells were suspended in an amount of trypsin solution equivalent to the original volume of the medium (1 ml. per Carrel flask), pipetted rapidly 10 to 20 times to break up any clumps, and then introduced into both chambers of a hemocytometer. When cell growth was heavy (1.5×10^6 to 2.5×10^6) 80 small squares in each side of the hemocytometer were counted, as in routine red-blood-cell counts. When fewer cells were present, all 400 of the small squares (25 large squares) in each half of the hemocytometer were counted.

Carrel flasks were inoculated with 0.1 ml. of cells (approximately 200,000), 0.8 ml. of standard medium, and 0.1 ml. of the desired dilution of inhibitor or Tyrode's solution. The inhibitors were made up in concentrated stock solutions in saline, sterilized by autoclaving, and diluted with Tyrode's solution when they were to be used.

The *p*H of the medium was tested at the start of the experiment and adjusted to about *p*H 7.6 to 7.8 by gassing the medium with 5 percent CO_2 in air where necessary. A change in medium was made after 3 days, the experiment was terminated after 5 or 6 days, and the final *p*H was noted.

A series of three to six Carrel flasks was used for each level of inhibitor studied, along with a parallel series of control cultures. The cells were removed from the glass by incubation with 0.25 percent trypsin for 30 minutes at 37° C. The cells from each group of flasks were pooled and counted. They were then washed three times with saline (0.85% NaCl) preparatory to chemical analysis, which included assays for total nitrogen, total fatty acids, and lipide phosphorus (*10–12*). Each experiment was repeated at least three times.

Results

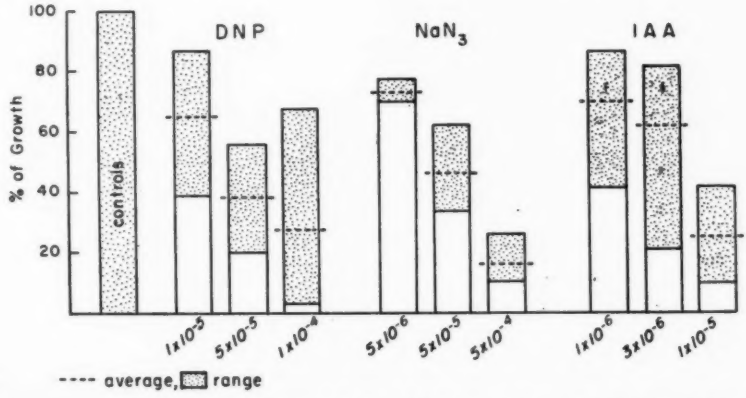
Measurable effects of DNP on the L cells could be determined at concentrations between 1×10^{-4} and 1×10^{-5} *M*. Toxic effects were marked at levels of 1×10^{-4} *M* and higher; the medium became quite acid (*p*H 6.1 *vs.* 6.9 in the controls); growth was strongly inhibited, and the resulting cells were usually heavily granular and frequently showed a bipolar type of pigmentation. At lower concentrations of DNP (5×10^{-5} and 1×10^{-5} *M*) the *p*H was comparable to that in the controls; cell growth was somewhat inhibited, and some slight increase in pigmentation or granulation was observed. However, no significant variations in the lipide composition of the L cells could be demonstrated.

Somewhat different effects were observed with IAA. At concentrations of 1×10^{-5} *M* growth was markedly inhibited and the cells were granulated or vacuolated, often rounded, and degenerative. The *p*H of these cultures averaged slightly higher than that of the corresponding controls. Growth of the L cells at this level of IAA was erratic and seemed to indicate the borderline of survival. Concentrations of 1×10^{-6} or 3×10^{-6} *M* produced little or no change in the normal appearance or chemical composition of the L cells but progressively inhibited their growth. At the most toxic level (1×10^{-5} *M*) marked changes in chemical composition occurred. A definite increase in the fatty acid : nitrogen ratio was noted without any corresponding increase in the lipide P content (table 1).

The effects of NaN_3 could be studied over a wide range (from 5×10^{-4} to 5×10^{-6} *M*). At concentrations of 5×10^{-4} and 5×10^{-5} *M*, the final *p*H of the medium became acid, usually about *p*H 6.5. Increases in fatty-acid content were striking with NaN_3 concentrations of 5×10^{-4} *M*. Inhibition of growth was relatively proportional to the concentration of the azide. Appreciable increase in the lipide P:nitrogen ratio was also apparent at levels of 5×10^{-4} and 5×10^{-5} *M*.

Changes in the appearance of the L cells were also marked. While the normal appearance of the cells in the absence of inhibitor shows a small, clear or slightly granular spindle-shaped cell, (fig. 1) the addition of NaN_3 at concentrations of 5×10^{-4} *M* provoked the appearance of predominantly large, abnormal round cells containing many dark granules. These cells appeared to be multinucleate (fig. 2).

Comparative growth of the cells in the control medium and in the presence of the various concentrations of the inhibitors is illustrated in text-figure 1. In every experiment the total number of L cells was greater in the control flasks than in those containing the inhibitors. It is important to note here that because of the variation in cell appearance and growth which may occur due to the age of the culture, the medium, or



TEXT-FIGURE 1.—Effects of some metabolic inhibitors on the multiplication of L cells.

TABLE 1.—*Effects of DNP, IAA, and NaN_3 on the lipide composition of strain L cells*

Inhibitor	Duration of expt. (*days*)	Concentration of inhibitor	L cells per flask	Final *p*H	Chemical composition of cells			
					Nitrogen (*μg. /flask*)	Fatty acid (*μ moles/mg. N*)	Lipide P (*μ moles/mg. N*)	Ratio fatty acid: lipide P
2,4-Dinitrophenol	6	$1\times10^{-4}M$	300, 000	6. 1	10. 9	3. 29	1. 65	2. 00
		$5\times10^{-5}M$	600, 000	6. 4	28. 4	2. 24	1. 29	1. 74
		$1\times10^{-5}M$	1, 000, 000	6. 9	39. 9	2. 33	1. 44	1. 62
		Control	1, 500, 000	6. 9	44. 6	2. 24	1. 39	1. 61
Iodoacetic acid	6	$1\times10^{-5}M$	400, 000	7. 2	23. 8	4. 32	1. 50	2. 88
		$3\times10^{-6}M$	500, 000	7. 0	36. 6	2. 40	1. 28	1. 86
		$1\times10^{-6}M$	600, 000	7. 0	37. 9	2. 34	1. 12	2. 09
		Control	1, 500, 000	6. 9	44. 6	2. 24	1. 39	1. 61
Sodium azide	6	$5\times10^{-4}M$	110, 000	6. 7	4. 7	7. 40	2. 68	2. 76
		$5\times10^{-5}M$	400, 000	6. 3	5. 7	6. 55	2. 56	2. 54
		$5\times10^{-6}M$	700, 000	7. 4	30. 6	3. 73	1. 87	2. 01
		Control	1, 000, 000	7. 4	30. 3	3. 46	1. 67	2. 04
Sodium azide	5	$5\times10^{-4}M$	90, 000	6. 7	3. 9	7. 48	2. 37	3. 16
		$5\times10^{-5}M$	400, 000	5. 8	13. 4	4. 69	2. 02	2. 32
		$5\times10^{-6}M$	600, 000	6. 9	31. 8	3. 61	1. 94	1. 86
		Control	800, 000	7. 0	39. 2	3. 25	1. 69	1. 92

particular lot of serum used, etc., it is essential that a control series be employed with each inhibitor experiment. Various samples of horse serum have shown different growth-promoting properties which may affect the appearance or chemical composition of the cultures.

Discussion

In various media in which a characteristic normal growth of L cells occurred, the ratio of fatty acid to lipide phosphorus has been found to average 2:1 or lower (*13*). The presence of sodium azide in concentrations of 5×10^{-4} to 5×10^{-5} *M* increased this ratio to 2.5:1 or higher. Both total fatty-acid and lipide-P levels were higher, with a disproportionate rise in fatty acids causing the shift in the ratio. Increases in fatty-acid content without changes in the lipide-P level were noted with IAA at concentrations of 1×10^{-5} *M*, while no statistically significant changes could be shown with DNP at the concentrations tested.

From these results it would seem that each of the three inhibitors acted in a different or specific manner in interfering with the metabolism of the L cells. No effect on the lipide metabolism could be demonstrated with DNP at concentrations which permitted the multiplication of the cells. Although some cellular pigmentation appeared in cultures containing DNP at a concentration of 1×10^{-4} *M*, the majority of the cells were normal in size and in nuclear appearance. Somewhat similar results were obtained by Verne (*14*). He found that DNP was toxic to 9-day chick-heart fibroblasts at dilutions of 1:30,000, while at dilutions of 1:90,000 no effects on growth were noted and the lipides showed no Feulgen-Verne reaction but responded in the same manner as the normal untreated cells.

Near-toxic levels of IAA (1×10^{-5} *M*) seemed to increase the neutral-fat content of the cells as demonstrated by a rise in the fatty-acid fraction alone. Morphologically the cells showed a definite granulation which was generally bipolar in appearance, but the preponderance of cells were of normal size and contained a single nucleus. At levels of 1×10^{-6} and 3×10^{-6} *M* IAA, the cells were found to be somewhat more granular, rounder, and larger than the corresponding controls.

With sodium azide a different picture was noted. At levels of 5×10^{-4} *M*, increases in both fatty acids and lipide P were found. As the photomicrographs show (figs. 1 and 2), the cells appeared large, mainly multinucleated, and were filled with deeply pigmented granules which suggested fat inclusions. At concentrations of 5×10^{-5} *M* the cells were less abnormal in appearance, although granular and somewhat pigmented. The corresponding changes in lipide content, as shown in table 1, were mostly due to increases in fatty acids without appreciable rises in the lipide-P levels.

Summary

2,4-Dinitrophenol, iodoacetic acid, and sodium azide were studied for their effects on the growth and lipide composition of the strain L mouse-fibroblast cells. The three respiratory inhibitors were found to affect the cells differently.

2,4-Dinitrophenol was toxic at concentrations of 1×10^{-4} *M*. Growth was inhibited at 5×10^{-5} *M* and 1×10^{-5} *M*, but at these concentrations the L cells were nearly normal in appearance, being only slightly granular or pigmented. No significant effect on the fatty-acid or lipide-P metabolism was noted.

Iodoacetate at levels of 1×10^{-5} *M* caused a marked increase in fatty-acid content without inducing a corresponding rise in lipide P. Morphologic changes in the cells were characterized by an increase in granulation without significant variation in size or nuclear appearance.

Sodium azide caused a striking rise in fatty acids and a smaller but parallel increase in lipide P at a concentration of 5×10^{-4} *M*. With 5×10^{-5} *M* sodium azide appreciable increases in fatty acids and lipide P were still noted. Morphologic changes, characterized by deeply granular or pigmented cells which were predominantly multinucleate, corresponded to the chemical changes noted.

Multiplication of Earle's strain L cells was reduced in the presence of all three inhibitors. The effect was approximately proportional to the concentration of the inhibitor used.

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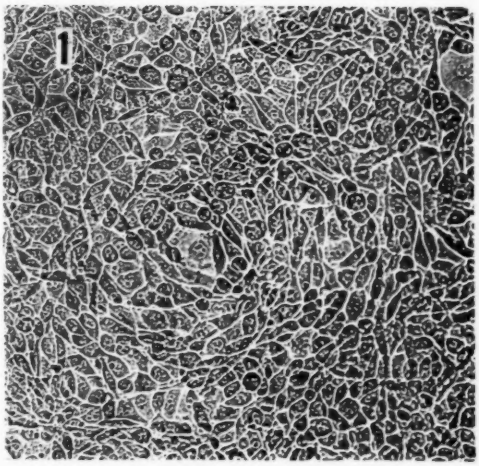
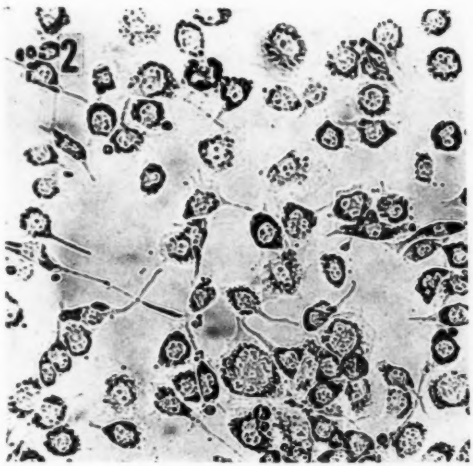
PLATE

PLATE 89

FIGURE 1.—Culture of strain L cells grown for 6 days in 40 percent horse serum, 40 percent mixture 199, and 20 percent Tyrode's solution. Growth is abundant and the cells are normal in appearance, being clear, slightly granular and triangular, or spindle shaped. × 145

FIGURE 2.—Culture of same cells grown in same medium containing sodium azide in a concentration of 5×10^{-4} *M*. Growth is relatively sparse. The cells are enlarged, often round, show a deep granular pigmentation in the cytoplasm, and are often multinucleate. × 145





These photomicrographs were taken by Mr. Victor Duran.

366462—56——25

Toxicity of Homologous Immune Serum to a Transplantable Tumor: Studies Using Phase Microscopy and Cinemicrography[1, 2]

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Transplantable tumors may induce an immunity in their host which leads to regression of the tumor and resistance of the host to subsequent transplants of the same tumor. This immunity to a transplantable tumor may be similar to the resistance phenomena displayed by some cancer patients to their tumor. In an occasional patient the resistance leads to spontaneous regression of the tumor and is associated, as pointed out by Stewart (*1*), with symptoms of an immune reaction.

In this study we consider the question: Are antibodies present in the blood serum of animals that have had a spontaneous regression of a homologous transplantable tumor? Many investigators have explored this problem but have not obtained consistent findings.

Methods used in the past to study homologous antisera have utilized the following techniques: 1) animal inoculation of tumor cells plus antisera, 2) serologic reactions against tumor antigens, 3) tissue culture of tumor cells in media containing antisera, and 4) morphologic studies of tumor cells in suspension.

Gorer (*2*) immunized C57 black mice to a leukemic tumor which originally developed in a strain A mouse. He added the immune sera to the leukemic cells and immediately inoculated the mixture into susceptible mice. The immune sera retarded or inhibited the growth of the tumor cells and were said, therefore, to contain protective or neutralizing antibodies. Similar antibodies were found by Burmester (*3*) in the serum of chickens immunized to chicken lymphoid tumor. The tumor cells were incubated with the antisera for 24 hours at 37° C. before testing by inoculation into chickens. Heating to 66° C. for 30 minutes had no effect on the activity of the antisera. Homologous protective antibodies were also observed by Kidd (*4*) against Brown-Pearce tumor cells from rabbits.

Antibodies homologous to tumor cells have been studied by serologic procedures. Complement-fixation tests were used to demonstrate an

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[3] The authors are indebted to Dr. Anna Goldfeder, who provided us with the Bagg tumor and to Dr. Kurt Stern for the Lewis lymphoma.

366462—56——26

antibody against antigens derived from the Brown-Pearce tumor by Kidd (*5*), Cheever (*6*), and by Lippincott and co-workers (*7*).

A few workers have been successful in demonstrating antibodies in homologous immune sera by the use of tissue culture. Lumsden, Macrae, and Skipper (*8*) found cytotoxins in the sera of rats hyperimmunized with Jensen rat sarcoma. These workers emphasized the rigid conditions needed for demonstrating the antibodies—blood had to be drawn 2 to 4 days after the last immunizing inoculation and serum had to be freshly prepared. The antiserum killed a tissue culture of tumor cells in 10 to 30 minutes but had no effect on cultures of normal rat connective tissue. Lumsden and Phelps (*9*) also reported cytotoxic properties in sera from rats with regressed or regressing Jensen rat sarcoma. Cytotoxic antisera in tissue culture were also demonstrated by Rerrich and Wettstein (*10*).

In recent years a few workers have used cell suspensions rather than tissue-culture methods to demonstrate the cytotoxicity of sera. Kalfayan and Kidd (*11*) observed structural changes in the cells of Brown-Pearce carcinoma as a result of incubation of tumor cells with immune homologous serum and complement. Similarly Okubo *et al.* (*12*) and Takeda (*13*) reported that the serum of immune rats caused vacuolar degeneration of the cells of Yoshida rat sarcoma.

In contrast to these scattered positive results, many investigators failed to find any evidence of antibodies in homologous serum as indicated in the reviews of Snell (*14*), Eichwald (*15*), and Prehn and Main (*16*). Many investigators would agree with the conclusion expressed by Harris (*17*) that ". . . cytotoxins, while active against heterografts, apparently do not appear in homoplastic transplantation."

In this laboratory, the method of unstained-cell counts (*18*) and of time-lapse cinemicrography (*19*) have been used previously in studying the cytotoxic effect of reagents on cells in suspension. These methods permit observations of the *in vitro* reactions between serum and cells. In preliminary studies (*20*), we have shown that sera from rats with regressed tumors were toxic to tumor cells. The present report supports our previous findings and analyzes the conditions required for demonstrating the cytotoxins.

Methods

The transplantable rat tumor of Bagg used in these studies was described by Goldfeder (*21*) and was diagnosed by Ewing as lymphosarcoma of reticulum-cell type. The growth originally developed and was carried on in rats of the Bagg strain. It was transplanted in this laboratory to random-bred rats of the Sprague-Dawley stock. The histologic, cytologic, and growth characteristics of the tumor have not changed during 200 consecutive transplants. The transplants grew progressively in about 65 percent of the animals. In another 25 percent the tumor grew and then regressed.

Suspensions of tumor cells in Ringer's solution were prepared by chopping up an excised tumor and filtering through 80-mesh Monel metal wire screen. By the method of unstained-cell counts, the suspension thus

prepared usually contained about 50 to 100 viable tumor cells per μml., 10 to 50 dead tumor cells, and a variable number of red blood cells and leukocytes. Usually blood cells were numerous in young tumors and were sparse in older, progressively growing tumors.

The tumor was transplanted by inoculating 0.1 ml. of the suspension subcutaneously into rats. In many rats the Bagg tumor grew to a mean diameter of 1 cm. or more and then regressed spontaneously. Most of these rats, when tested, were immune to a second implant of the tumor. Immune and normal control rats were anesthetized with ether, and blood was obtained by cardiac puncture. The immune blood was obtained weeks and months after the regression of the tumor.

For photography and microscopic study, the suspension used contained 1 viable tumor cell per μml. in a medium consisting of 50 percent fresh immune or normal rat serum in Ringer's solution. A drop of the suspension (0.04 ml.) was placed between two large coverslips separated from each other by a metal ring 0.8 mm. in thickness. The cells in the chamber rapidly settled to form a single layer of cells. The preparation was placed on the stage of an inverted metallurgic microscope with the objective below the stage and the condenser above. Part of the microscope, including the stage, was enclosed in a lucite incubator which maintained the temperature of the preparation at 37° C. The microscope was equipped with a 16-mm. movie camera and time-lapse equipment for obtaining exposures at intervals. The illumination was provided by a high-pressure mercury-vapor lamp. A shutter was placed between the light and the microscope mirror and was tripped by a solenoid for exposure of the film. The shutter minimized the exposure of the cells to light.

A 20× phase objective B minus L was usually used for the photography of the tumor cells. One frame of the movie film was exposed every 6 seconds with an exposure of 1/25 second. The magnification on the film was 76×.

Observations were made of these preparations to determine the difference between tumor cells suspended in normal rat serum and in immune rat serum. Attention was paid to the length of survival of cells and to the morphologic changes preceding cell death. Additional experiments were done to determine the titer and specificity of the antibody and the effects of heat and complement.

Bagg Tumor Cells in Immune Serum

Bagg tumor cells in 50 percent immune rat serum were studied by time-lapse cinemicrography with bright-field illumination. The tumor cells appeared translucent and finely granular (fig. 1). Only an occasional cell showed ameboid motion. Immediately after the addition of the immune serum to the cellular suspension, most cells were round and inactive with a sharply outlined cell border. The nucleus was not visible. A slow, rhythmic movement of the peripheral zone of the cell was seen in the projected film. The cells were evidently viable but in an early stage of degeneration. Gradually the cells developed large prominent nuclei with

thin nuclear walls and large nuclei (cell A, fig. 2). The interior of the nuclei appeared clear and homogeneous, and the cytoplasm appeared coarsely granular. These degenerated cells with conspicuous nuclei gradually or suddenly became indistinct and transparent with soft, barely visible outlines (cells A, C, and D, fig. 4). The flattened ghost cells continued to undergo further changes toward increasing transparency until they became almost completely invisible.

The morphologic changes in the tumor cells may be termed *cytolysis*. The process was similar to the hemolysis of red blood cells when observed by time-lapse cinemicrography. Thus Bagg tumor cells in immune rat serum underwent preliminary degenerative changes and finally cytolysis.

Tumor cells in immune serum were also studied by phase cinemicroscopy. As soon as the preparation could be photographed, degenerative changes were present. At first the cells were spherical with prominent outlines. Usually the cell contained a dark central mass, apparently an artefact caused by the thickness of the cell (cell I, fig. 5). The nucleus gradually became visible and contained a clear, bright central area and coarse, dark granules attached to the nuclear wall (cell L, fig. 12). The next step in degeneration as seen with phase microscopy, was sudden and dramatic. The nuclear membrane collapsed, liberating the nuclear contents, which then appeared as a clear mass with chromatin granules in the cytoplasm (cell L, figs. 12–14). Following this, the cell slowly underwent *post-mortem* changes. The end result was a small, poorly outlined cell with many coarse granules (cell J, fig. 10).

With some immune sera, many tumor cells were killed almost immediately, and all the cells were dead and morphologically altered within 1 hour. In experiments with time-lapse cinemicrography, the immune sera were selected because of their low titer of activity in order to permit observation of the degenerative changes. In figure 1, all the cells showed degenerative changes but were still viable 11 minutes after the addition of the immune sera. After about 1 hour, only 9 out of 15 cells were alive (fig. 4). With the low-titer serum used in figures 5 to 16, about half of the cells were alive after 1 hour (fig. 8), a few cells were alive after 2 hours (fig. 15), and 1 cell (cell M) survived 6 hours (fig. 16).

Both phase and bright-field microscopy showed that the immune serum caused the tumor cell to round up and to develop a large, vesicular nucleus. These changes were followed by a sudden rupture and collapse of the nuclear wall, according to phase microscopy, and by an increased transparency or cytolysis of the cells, according to bright-field microscopy. Ghost cells resulting from the death of tumor cells in immune serum were more clearly visible by phase microscopy than by bright-field illumination.

Bagg Tumor Cells in Normal Rat Serum

In control cinemicrographic experiments the reactions between tumor cells and normal serum were studied. Ameboid activity was characteristic of many cells in normal serum but was seen only in a few rare cells in immune serum. Cells in ameboid motion had one or more anterior

pseudopods which appeared light by phase microscopy and a single posterior tail which appeared dark (cell S, fig. 19 and cell T, fig. 17). These cells had distinct, large nuclei with large chromatin and nucleolar masses. Many cells maintained normal appearance and activity for 24 hours.

Degenerative changes began at irregular intervals. Ameboid activity ceased and the cell became round. Later changes in the shape of the cell occurred rapidly and multiple lobules developed (cell W, fig. 23). With phase microscopy each lobule seemed to have a small, clear vacuole surrounded by a thin, dark ring, probably of chromatin material (cell N, fig. 19). Lobulation continued for about a half hour. Gradually the changes in shape became less active, the lobules retracted, and the cell became spherical, motionless, and dead. Frequently the dead cell appeared bright and doubly refractile (cell N, fig. 20). The dead cells underwent *post-mortem* changes, becoming small and granular. Death of the tumor cells by lobulation was similar to that observed for normal rabbit lymphocytes (*19*). This is not surprising, since the Bagg tumor cell is a malignant lymphocyte.

The final stage observed in the cells in immune and normal sera were similar, namely, they were dead and had a small, granular appearance. However, the survival period of tumor cells in immune serum was short and in normal serum it was long. More striking were the degenerative changes which preceded cell death. In immune serum, the tumor cells underwent rounding up, vesiculation, and rupture of the nucleus; in normal serum, the cells underwent lobulation and multiple vacuolation.

A few incidental observations were made on the behavior of tumor cells in normal serum during the study of the time-lapse movie film. The cells were polarized with anterior and posterior ends. The cells traveled in straight lines and usually pushed aside small obstacles such as red blood cells. When they came in contact with stationary obstacles, they veered off at an angle. Occasionally, the cell rotated 180° reversing its direction of motion (cell V, figs. 26–28).

Tumor cells coming in contact during ameboid activity did not affect each other except as obstructions. In some preparations, the viable tumor cells had a tendency to adhere to various objects, such as red blood cells, lymphocytes, and dead and even living tumor cells. These objects usually adhered to the tail and were dragged by the ameboid tumor cell. Figures 26 to 28 show a dead tumor cell (W) which had become adherent to the tail of an active tumor cell (V) and remained adherent for more than 1½ hours. In some normal sera, two or more viable tumor cells adhered to each other by their tails. In these cases, the cells had a tug of war in their attempt to move in opposite directions. Frequently, one cell seemed stronger and pulled the other cell behind it.

Factors Affecting the Cytotoxicity of Immune Serum

Quantitative data on reagents used.—The morphologic changes described previously are based on the use of sera which were obtained from 78

immune rats. These rats had been inoculated with a suspension of Bagg tumor cells and had developed a tumor which regressed spontaneously. The tumors were measured three times a week, and the size of the tumor was expressed as the mean diameter, or the geometric mean, of the three dimensions of the growth. When the tumors reached their maximum size, the mean diameters varied from 11 to 35 mm., with a median of 21 mm. The time interval between inoculation and maximum size had a median of 11 days and varied from 7 to 14 days. The tumors regressed rapidly and completely in most of these rats, although in a few animals a small residual nodule persisted for a long time so that the exact date of regression was ill-defined. The time between inoculation and regression varied from 16 to 67 days, with a median of 43 days.

Of the 78 immune rats, 35 were reinoculated with a tumor-cell suspension to test and increase the immunity of the animals. A few reinoculated rats developed a small nodule which promptly regressed, but most of the rats did not develop any palpable growth. The 35 reinoculated immune rats were bled one to three times to give 54 immune sera. An additional 49 immune sera were obtained from the other 43 rats which had not been tested by reinoculation.

Since the time of regression is somewhat indefinite, it may be preferable to indicate the time of bleeding from the date of inoculation of the original tumor. The 103 immune sera were obtained 20 to 222 days following the inoculation of the original tumor. The median time interval was 58 days. The 43 immune rats that were not reinoculated were bled 20 to 175 days, with a median of 51 days, after inoculation of the tumor.

Normal sera for controls were obtained from 42 rats of the same Sprague-Dawley stock used for inoculating the Bagg lymphosarcoma and developing immune animals. The 42 normal and 103 immune sera were first tested individually. Usually 4 to 6 immune and 1 or 2 normal sera were tested simultaneously against 1 tumor-cell suspension.

Twenty-nine cell suspensions were used for testing the sera and were obtained from 29 Bagg lymphosarcomas. The tumors were used 8 to 17 days after inoculation (median 12.5 days) and measured 16.0 to 30.6 mm. in mean diameter (median 26.4 mm.). The 0.04 ml. of test preparation contained 0.01 to 0.02 ml. of normal or immune sera and 22,000 to 79,000 viable tumor cells, 12,000 to 56,000 dead cells, and 18,000 to 198,000 red blood cells (medians 4,000 viable, 21,000 dead, and 72,000 red blood cells, respectively). The preparations were examined by phase microscopy after 4 hours of incubation.

With the 103 immune sera, 70 (68.0%) gave a positive immune reaction as indicated by the development of vesicular nuclei, followed by collapse of the nuclear walls and death of 50 percent or more of the tumor cells within 4 hours of incubation. A slightly positive reaction (nuclear changes in about 25 percent of the tumor cells) was observed in 9 sera and a negative reaction in 8 (12.6%). In contrast, the immune cytocidal reaction was not observed with the 42 normal rat sera tested in this series of experiments nor with many other normal rat sera used as controls in

other experiments. The tumor cells in all the normal sera remained viable and motile during the 4-hour period of incubation. It may be concluded that the regression of the Bagg lymphosarcoma is associated with the appearance of a cytotoxic agent in the blood serum of the host.

The present experiments were chiefly concerned with testing normal and immune sera and with studying the properties of the antisera. A few incidental observations were made on the development of the immune reactions in rats. As mentioned previously, 43 rats were not reinoculated after the regression of their tumor. These rats provided 49 immune sera of which 65.3 percent were positive, 18.4 were slightly positive, and 16.3 percent were negative. The corresponding figures for 54 immune sera from 35 reinoculated rats were 70.4, 20.4, and 9.3 percent. In rats with only a single inoculation of tumor, 1 positive serum was obtained 3 months after the inoculation of tumor and 1 slightly positive serum 5 months after the inoculation. These findings indicate that the regression of the Bagg lymphosarcoma is usually followed by the appearance of antibodies in the serum and that antibodies persist for a prolonged period of time.

Antibody titer.—To determine the antibody titer, dilutions of the immune serum were added to the tumor-cell suspension. The mixtures, in 0.04 ml. amounts, were placed between two large coverslips and sealed with paraffin. The preparations were incubated at 37° C. and observed with phase microscopy. It was possible to identify ameboid tumor cells, dead cells, and intermediate degenerating cells. Identification of the cells was not difficult after experience gained with time-lapse cinemicrography.

In one experiment (table 1) slight toxicity was observed when the immune serum had a final dilution of 1:128, and this was considered the titer of the antiserum. A few sera had slightly higher titers (1:256). In control experiments this toxic reaction was not observed with any of the normal sera, even in the maximum concentration of 1:2.

Specificity of the antibody.—The same antisera were tested against the cells from Walker carcinosarcoma 256, Lewis lymphosarcoma (*22*), and rat lymphocytes prepared from normal rat thymus. These cells remained viable during a 4-hour observation period and appeared the same whether incubated with immune or normal rat serum. Thus the antibodies were not toxic to cells from Walker carcinosarcoma, Lewis lymphosarcoma, or rat thymus (table 1).

Effect of heat and addition of complement.—Immune sera were heated to 56° C. for a half hour and then tested for toxicity (table 1). The heated immune serum did not kill the tumor cells but caused clumping of apparently viable cells. The tails of tumor cells were seen to adhere to each other and the cells in the clumps remained viable for many hours. This type of agglutination was observed in nearly all heated immune sera and in a few normal sera. Addition of fresh normal rat serum to a mixture of tumor cells and heated immune serum restored the toxicity more or less completely. As seen in table 1, the heated immune serum had a

TABLE 1.—*The effect of normal and immune (to Bagg lymphosarcoma) rat sera on the agglutination and viability of Bagg lymphosarcoma cells and other cells as determined by phase microscopy. Note that the Bagg lymphosarcoma cells but not other cells were killed in 4 hours, apparently by a homologous antibody plus complement**

Rat sera tested	Cells derived from:	Other reagents	Agglutination in 4 hours	Cells dying during 4 hours' observation	Titer of antiserum
Fresh normal.....	Bagg lymphosarcoma	None	Rare	None	
Fresh immune.....	Bagg lymphosarcoma	None	0	All	1:128
Fresh immune.....	Lewis lymphosarcoma	None	0	None	
Fresh immune.....	Walker carcinosarcoma	None	0	None	
Fresh immune.....	Rat thymus	None	0	None	
Heated immune....	Bagg lymphosarcoma	None	+++	None	
Heated immune....	Bagg lymphosarcoma	Heated normal serum	+++	None	
Heated immune....	Bagg lymphosarcoma	Fresh normal serum	0	All	1:128

**Procedure.*—The preparation consisted of 0.4 ml. of suspension which contained 0.1 ml. of the rat serum tested (column 1), 0.1 ml. of other reagents (column 3), and about 40,000 viable tumor cells or 300,000 viable cells from the rat thymus. It was incubated at 37° C. for 4 hours and examined by phase microscopy for agglutination and cell viability as indicated by motility and morphology. The titer of the antiserum was studied in subsequent experiments by determining the amount of serum that killed about half the tumor cells in 4 hours.

titer of about 1:128, which was the same titer as that of the antiserum before heating. Presumably complement is required for the cytotoxic action of the antibody in immune serum.

Adsorption studies.—To determine the specificity of the immune serum, various types of cells were added to heated immune serum and the mixtures were incubated at 10° C. for 2 hours in a shaker. A large volume of cells was used for these adsorption studies. After incubation the cells were precipitated by centrifugation and the supernatants were tested for immune antibody by the addition of a tumor-cell suspension and fresh normal serum. The results are shown in table 2.

TABLE 2.—*Adsorption of rat antisera (to Bagg lymphosarcoma) with various types of cells. Note that the antibody was removed from solution by viable Bagg lymphosarcoma cells but not by other viable cells and usually not by dead Bagg lymphosarcoma cells*

Adsorption procedure*			Results of testing procedure	
Type of cells used for adsorption of antibody	Treatment of cells before adsorption	Viability of cells during adsorption	Titer of immune sera against fresh Bagg lymphosarcoma cells	
			Before adsorption	After adsorption
Bagg lymphosarcoma	None	Viable	1:64	<1:8
Lewis lymphosarcoma	None	Viable	1:32	1:32
Walker carcinosarcoma	None	Viable	1:32	1:32
Rat thymus	None	Viable	1:64	1:64
Rat spleen	None	Viable	1:32	1:32
Bagg lymphosarcoma	56° C., 30 min.	Dead	1:128	1:128
Bagg lymphosarcoma	4° C., 13 days	Dead	1:64	1:64
Bagg lymphosarcoma	Ether	Dead	1:64	1:64
Bagg lymphosarcoma	HCl	Dead	1:64	1:64

**Procedure.*—Aliquots of 0.6 ml. of immune sera were adsorbed with 2 to 3 million untreated or treated cells at 10° C. for 2 hours. The viability of the cells used for adsorption was determined by the method of unstained-cell counts. The titer of the immune serum before and after adsorption was obtained as in table 1.

In a typical experiment the antibody titer of the immune serum was 1:64. After adsorption with viable Bagg cells, the supernatant was not toxic to the tumor cells in the maximum concentration tested (1:8, table 2). In this and other experiments the antibody was more or less completely removed by adsorption with Bagg lymphosarcoma cells.

In further work, viable Bagg lymphosarcoma cells were allowed to adsorb the antibody at 10° C. The cells were then tested, after centrifugation, by the addition of 50 percent fresh or heated normal serum and incubation at 37° C. for 4 hours. The cells in fresh normal serum were killed but not those in heated serum. Evidently the tumor cells adsorbed the antibody and were killed on addition of the complement in the fresh normal serum.

The cells of other rat tumors were used for adsorption to test the specificity of the antibody. The tumors used were the Lewis lymphosarcoma (*22*) and the Walker carcinosarcoma 256. The titer of the immune serum before and after adsorption with these two tumors was

the same (1:32). Both failed to remove any of the antibody to the Bagg lymphosarcoma.

To test the tissue specificity of the immune serum, lymphocytes derived from the thymus and spleen of the Sprague-Dawley rat were tested for capacity to adsorb the antibody to the Bagg lymphosarcoma. The titer of the sera was found to be the same before and after adsorption (table 2). It should be noted, however, that the normal cells were derived from random-bred rats of the Sprague-Dawley strain, while the original Bagg lymphosarcoma arose in a rat of the Bagg inbred strain. It appears that the normal lymphocytes tested did not adsorb the antibody to the Bagg lymphosarcoma.

Further adsorption studies were done with Bagg tumor cells that were killed by various methods. The antibody was not adsorbed by tumor cells killed by heat (56° C., 30 minutes), prolonged refrigeration (4° C., 13 days), ether, or hydrochloric acid. Dead tumor cells did not adsorb the antibody.

Discussion

This study indicates that spontaneous regression of the Bagg lymphosarcoma is associated with the appearance of an antibody in the blood serum of the host animal. The antibody agglutinated the tumor cells and was removed from the solution by viable tumor cells. The antibody plus rat complement produced a type of death characterized by vesiculation and rupture of the tumor-cell nucleus, which was not observed with other toxic reagents studied in this laboratory. Bessis (*23*) observed similar intracellular changes in human leukocytes exposed to heterologous rabbit antibodies. On the other hand, Kalfayan and Kidd (*11*) found that homologous immune serum did not produce any remarkable nuclear change in cells of the Brown-Pearce carcinoma. By careful staining methods they showed that the tumor cells exposed to immune serum suffered swelling and edema which involved the cytoplasm, the mitochondria, and the nucleolus.

The present experiments showed that immune sera produced definite cytologic changes, and the experiments were readily reproducible under the given conditions. Certain factors, on occasion, prevented the demonstration of cytotoxicity. The inhibiting factors observed in these and other experiments were: 1) a relative excess in the number of tumor cells, 2) an insufficient amount of complement, and 3) the presence of anticomplementary factors.

It is possible to speculate whether a rat, after regression of a Bagg tumor, had enough antibodies in its serum to kill the tumor cells in a second implant. In the present studies, 0.04 ml. of final suspension contained 0.01 ml. of immune serum and 1 tumor cell per μml. The antibody in this preparation killed the cells in less than 1 hour. In other words, 0.01 ml. of serum or about 0.02 ml. of blood rapidly killed 40,000 tumor cells. The inoculum used in this laboratory for transplantation of tumor consisted of 0.1 ml. of a su pension containing as many as 100 viable

cells per μml., or a total number of 10,000,000 cells. This number of viable cells would require 5 ml. of blood, and this amount of blood should be available in a rat weighing 150 gm. Thus the immune rat had sufficient antibodies in its serum to inhibit the growth of the implant. The regression of a large tumor could probably not be attributed to the amount of antibodies in the serum. Possibly the continued production of antibodies by the rat would be sufficient to cause regression. Thus, immunity to Bagg transplantable tumor is associated with immune bodies in the blood serum.

The next question that arises is—what is the antigen that reacted with the antisera? Even though both the tumor cell and the sera were from the "rat" or homologous, it is probable that the tumor cells had several or many antigens which stimulated the production of diverse antibodies in the immune rat. Since the antibodies were more or less completely adsorbed by viable tumor cells, some of the antigens were, presumably, on the surface of the cells.

The hypothesis of the localization of the antigens on the cell surface is, however, very incomplete. It does not explain the morphologic changes of nuclear vesiculation and rupture observed with antisera. It does not aid in determining to what extent the reagents are strain, organ or tumor antigens, and antibodies. The *in vitro* methods seem particularly useful in studying these problems.

Summary

The cells of Bagg transplantable rat lymphosarcoma were incubated *in vitro* with fresh serum from normal rats and with serum from rats with regressed tumor. *In vitro* observations were made by time-lapse cinemicrography and phase and bright-field microscopy. Blood sera from rats with regressed tumors were cytotoxic to Bagg lymphosarcoma cells. The degenerative changes produced by the toxic factor consisted of rounding up of the tumor cell, followed by swelling and vesiculation of the nucleus, and finally rupture and collapse of the nuclear wall. In contrast, the degenerative changes which preceded death of tumor cells incubated in normal serum were characterized by actively changing lobulations of the cell and multiple vacuoles in the nucleus. Tumor cells survived incubation in immune serum for about 1 hour and in normal serum for 12 hours and more.

The toxic factor was adsorbed from immune serum by viable but not by dead tumor cells of Bagg lymphosarcoma. Other tumor cells and normal cells of the rat were not killed by the immune sera and did not adsorb the toxic factor from the antisera. The immune sera was inactivated by heating to 56° C. for 30 minutes and the activity was restored by the addition of normal rat serum.

Conclusions

Sera from rats with regressed Bagg lymphosarcoma have an antibody against the cells of this tumor. The antibody by itself caused agglutina-

tion of Bagg tumor cells. The antibody plus rat complement rapidly killed the tumor cells and produced cytologic changes which were characteristic and pathognomonic of the immune reaction. The antibody was specific, had no effect on, and was not adsorbed by the other cells tested. The antibody is associated with the immunity in rats with spontaneous regression of the Bagg lymphosarcoma.

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PLATE 90

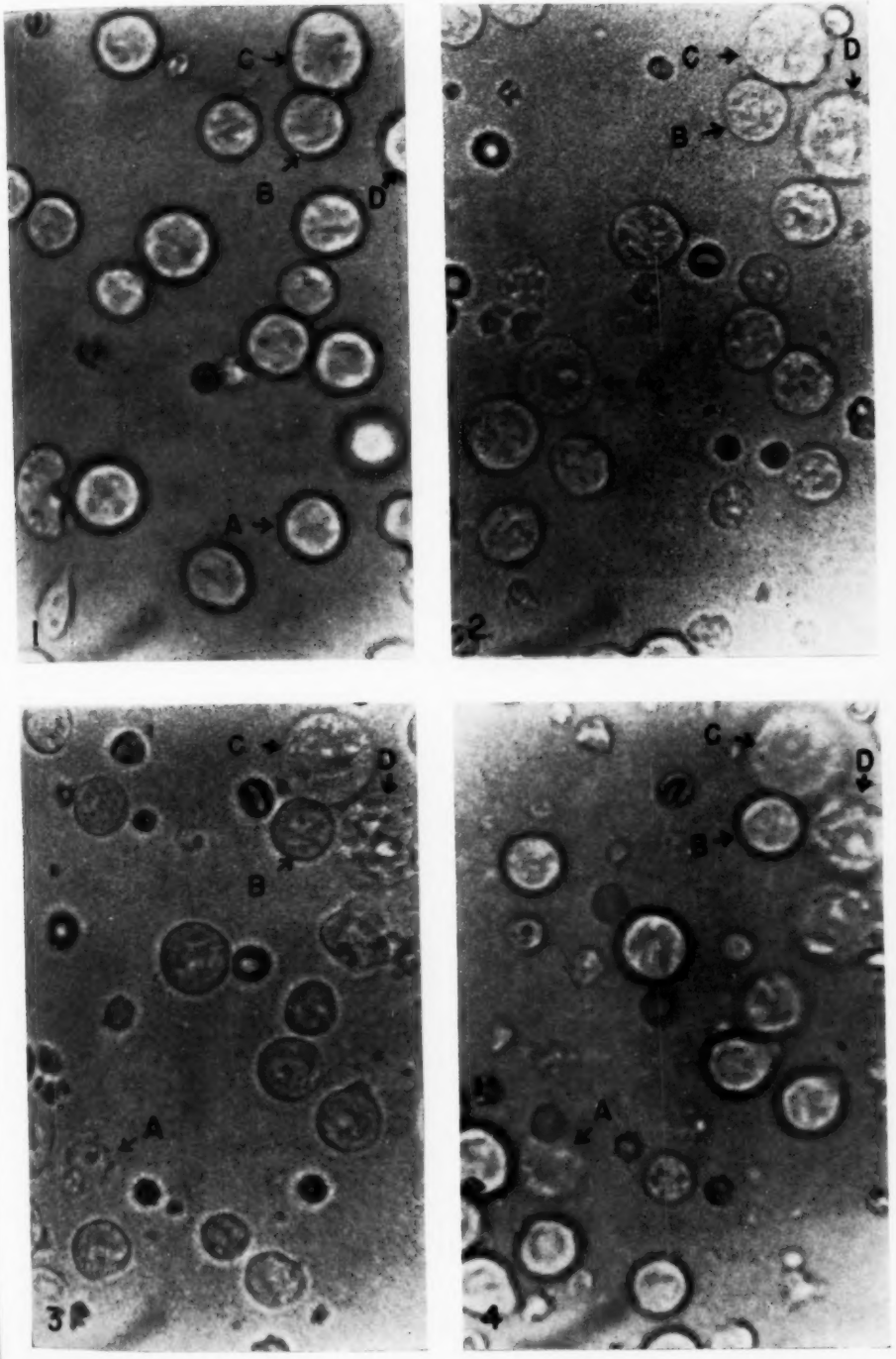
Sequence showing degenerative changes in Bagg lymphosarcoma cells incubated in immune rat serum as seen with bright-field illumination. A low-titer serum was used in order to permit the observation of cytologic changes. The objective was 20× apochromatic. The final magnification in this and subsequent plates is × 800. Prints in this and subsequent plates were made from 16 mm. cinemicrographic films.

FIGURE 1.—Most of the 17 cells in the figure are in the first stage of degeneration and are rounded up, with thick, circular shadows around the cell outlines. In the projected film, they are seen to be viable as indicated by the rhythmic motion of the peripheral cytoplasm. The cells are slightly out of focus in order to give depth to the picture and to aid in evaluating the thickness of the cells (*cf.* fig. 4). A red blood cell is present in the center of the field. Time: 11 minutes after preparation.

FIGURE 2.—The nucleus of cell A is undergoing vesiculation. The cell has increased in size; the nucleus is prominent and has a large nucleolus, clear nucleoplasm, and a thin nuclear wall. The cytoplasm has many coarse granules. Similar changes are occurring in cells C and D. Time: 33 minutes.

FIGURE 3.—The final stage of degeneration is illustrated by cells A, C, and D. They are cytolysed and transparent. Some granules and a large nucleolus are still visible in cell D. Time: 49 minutes.

FIGURE 4.—The tumor cells are slightly out of focus and this accentuates the difference between the viable cells and the cytolysed ghost cells. The viable cells such as B are similar to those seen in figure 1. The ghost cells, A, C, D, and others, are transparent and have soft outlines. The print shows 9 viable cells and 6 ghost cells. The red blood cells are in good focus and are normal and not hemolyzed. Time: 69 minutes.



Schrek and Preston

366462—56——28

PLATE 91

Sequence showing degenerative changes of Bagg lymphosarcoma cells incubated in 50 percent immune serum as seen with phase microscopy (B minus L phase objective, 20×).

FIGURE 5.—Cells F, G, and H are dead. Most of the cells such as J, K, and I are rounded up and appear to have bright peripheral zones and dark centers. These morphologic characteristics indicate early degenerative changes. Cell E has a posterior tail which suggests motility but in the projected film the cell shows only rhythmic oscillations. A few red blood cells are present and nearly all are normally biconcave with clear centers. Time: 37.5 minutes after preparation.

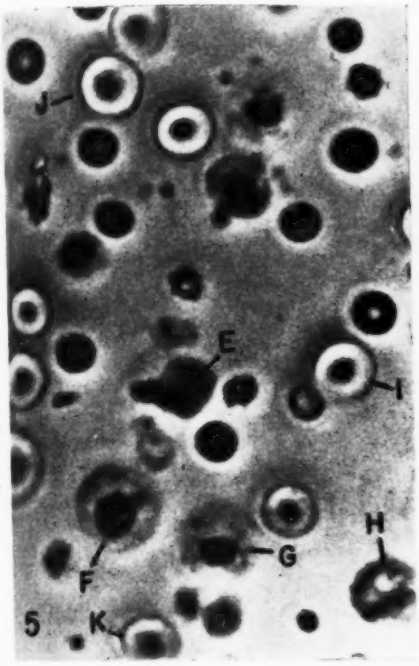
FIGURE 6.—The nucleus of cell J is beginning to be visible. Time: 59.8 minutes.

FIGURE 7.—Cell J has developed a large vesicular nucleus. The nucleolus and chromatin granules are attached to the prominent nuclear wall. At one point the nuclear wall appears ruptured. Time: 61.5 minutes.

FIGURE 8.—The nucleus of cell J has collapsed and the cytoplasm contains extruded nuclear material, namely dark granules and clear vacuoles. Time: 61.9 minutes.

FIGURE 9.—Cell J is beginning to undergo *post-mortem* changes but the cell is still large with a few dark chromatin masses in the pale cytoplasm. Cell L is rounded up and has a bright peripheral zone and dark center. It shows typical early degenerative changes. Time: 63.0 minutes.

FIGURE 10.—Cell J shows more advanced *post-mortem* changes as is indicated by its small size and the small, dark masses in the cytoplasm. Time: 98.6 minutes.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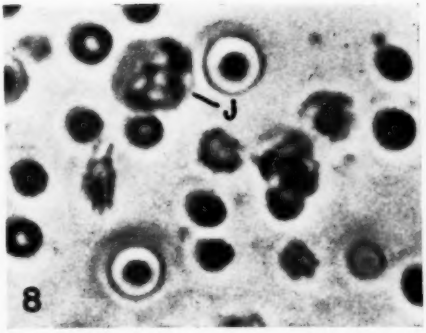
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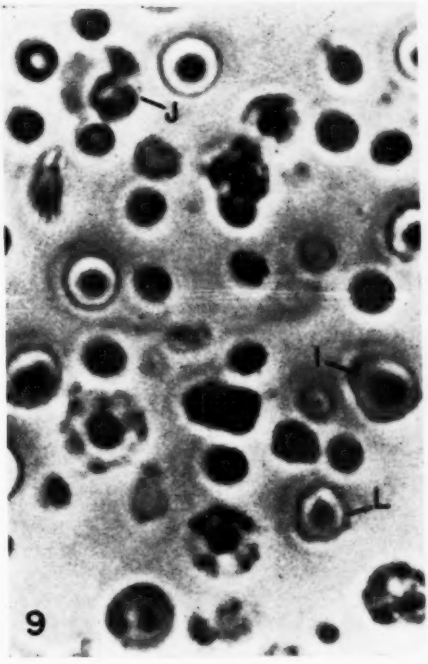
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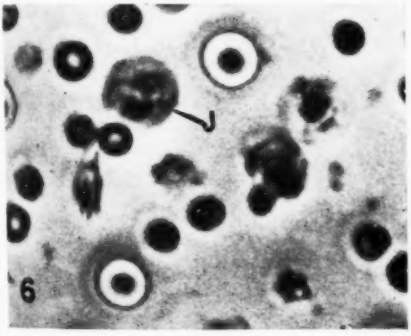
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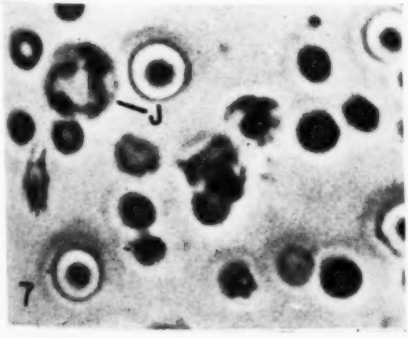
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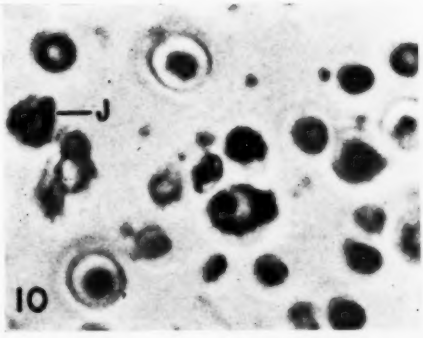
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PLATE 92

Continuation of sequence presented in plate 91.

FIGURE 11.—Cell L shows slight irregularity in shape as contrasted to its roundness in figure 9. Time: 65.0 minutes.

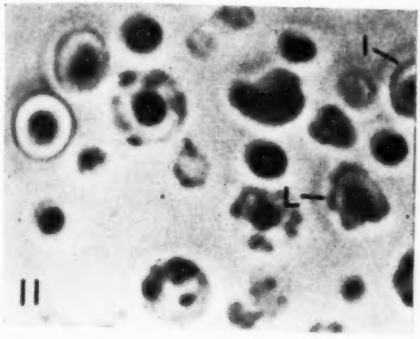
FIGURE 12.—The nucleus of cell L has become large and vesicular with dark masses attached to the nuclear wall. Time: 98.6 minutes.

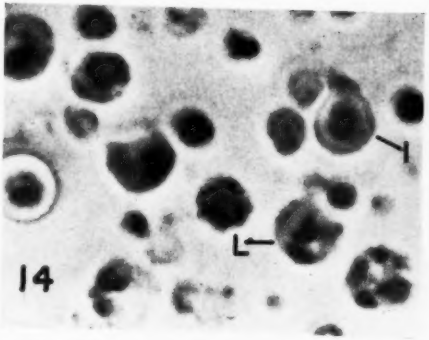
FIGURE 13.—The nucleus of cell L begins to collapse. Time: 98.7 minutes.

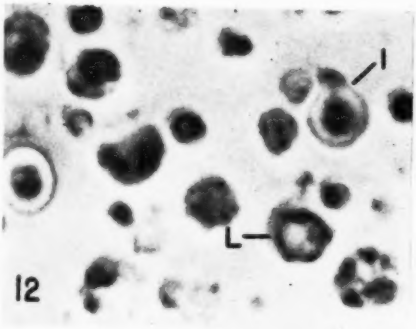
FIGURE 14.—The nucleus of cell L is collapsed with large, dark nuclear masses in the dark cytoplasm. The collapse of the nucleus has occurred in 12 seconds. Time: 98.8 minutes.

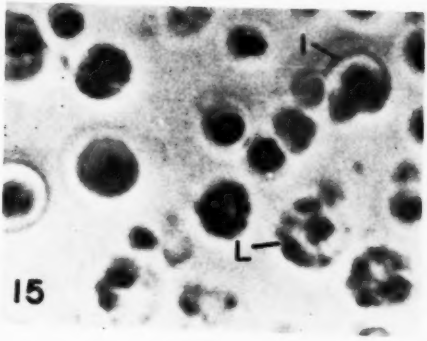
FIGURE 15.—The dead cell L is still large with scattered, dark granules in a transparent cytoplasm. The transparency of the cytoplasm suggests early *post-mortem* changes. Time: 117.6 minutes.

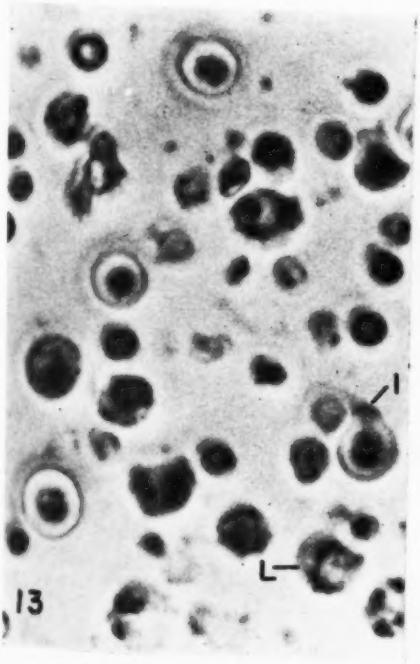
FIGURE 16.—This print represents a different microscopic field from those represented in figures 5 to 15. This field was selected because it contained a normal viable cell M which has a posterior tail and anterior pseudopods. This cell is in ameboid motion in the projected film. The small, dark granular cells are dead with advanced *post-mortem* changes. The red blood cells are spherical in shape and are not lysed by the immune serum. One red blood cell in the *upper left corner* is still biconcave. Time: 365.0 minutes.

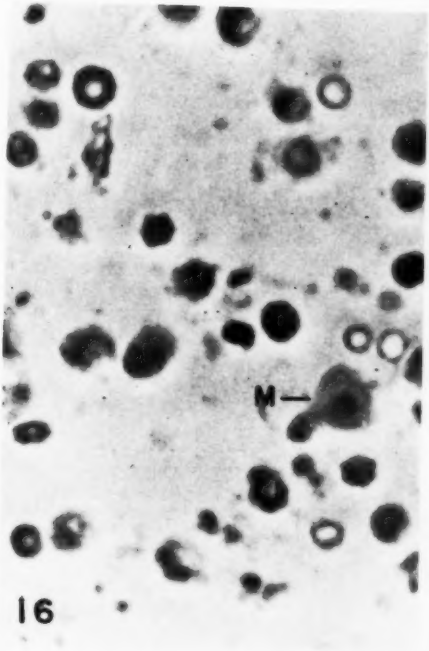












Schrek and Preston

366462—56——29

PLATE 93

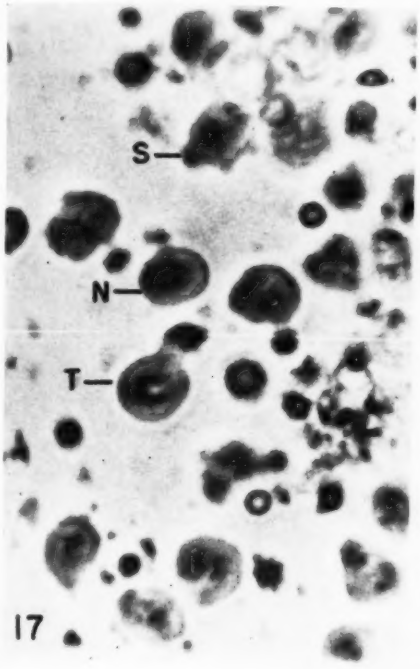
Sequence showing degeneration of Bagg lymphosarcoma cells in normal serum—photographed with phase optics (20× B minus L objective).

FIGURE 17.—Cell T has a single, blunt, posterior tail which appears dark by phase microscopy and bright under bright-field illumination (*cf.* fig. 28, cell V). Cell S has a posterior tail and two faint, delicate, blunt pseudopods. Time: 5 hours and 44 minutes.

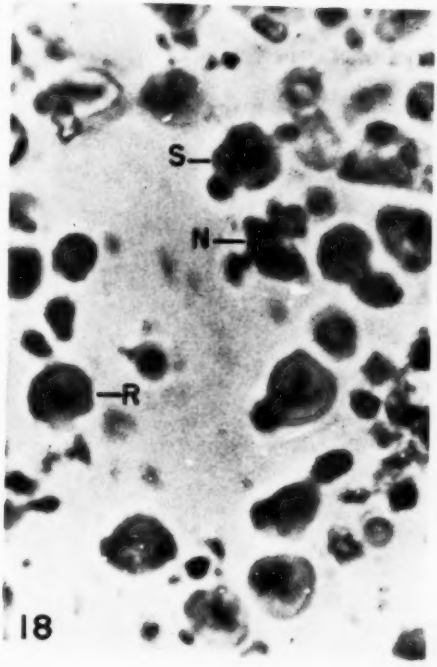
FIGURE 18.—Cell N is beginning to undergo typical degenerative changes characterized by multiple lobulations of the cell and, in the projected film, rapid changes in shape. Time: 5 hours and 59 minutes.

FIGURE 19.—Cell N continues to undergo degenerative changes, the most striking of which is the appearance of multiple vacuoles, each surrounded by a dark chromatin ring. The anteriorly placed pseudopods of cell S are characteristic of cells exhibiting ameboid motion. Time: 6 hours and 17 minutes.

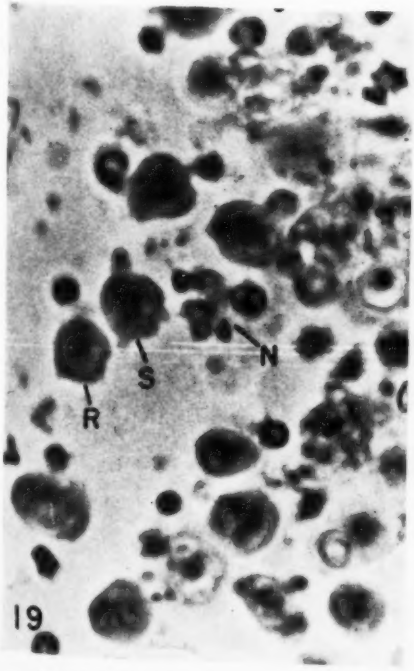
FIGURE 20.—Cell N is now dead and appears irregularly round with a large, bright center. Cells R and S, which have appeared normal in the previous figures, are undergoing lobulation. Time: 7 hours and 3 minutes.



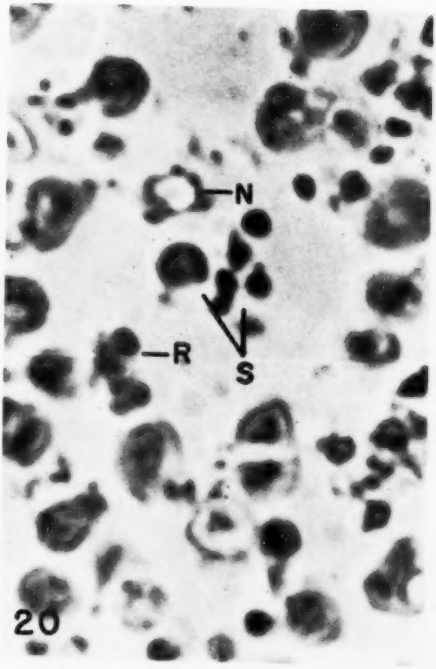
17



18



19



20

PLATE 94

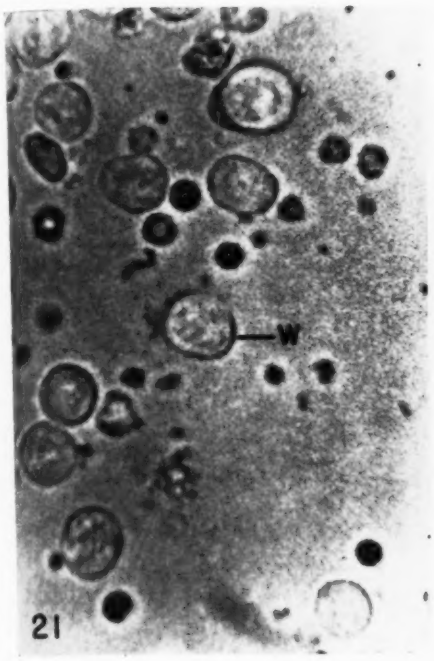
Sequence showing degeneration of Bagg lymphosarcoma cells in normal serum photographed with bright-field illumination (objective 20× apochromatic).

FIGURE 21.—The tumor cell W is round with coarse granules. It has just stopped moving and has retracted its pseudopods and tail. Time: 11 hours, 3 minutes.

FIGURE 22.—Cell W has developed a large vacuole and a few small lobules. Time: 11 hours, 57 minutes.

FIGURE 23.—Cell W has many lobules and, in the projected film, the cell is seen to undergo rapid changes in shape. Cell V is just entering the microscopic field and is moving toward two leukocytes. Time: 12 hours, 14 minutes.

FIGURE 24.—Cell W is blurred and partly out of focus because of the rapid changes in shape of the cell. Cell V is seen to be a large tumor cell in ameboid motion with a broad posterior tail. The cell has pushed aside the two leukocytes and has hit a red blood cell. Time: 12 hours, 37 minutes.


W
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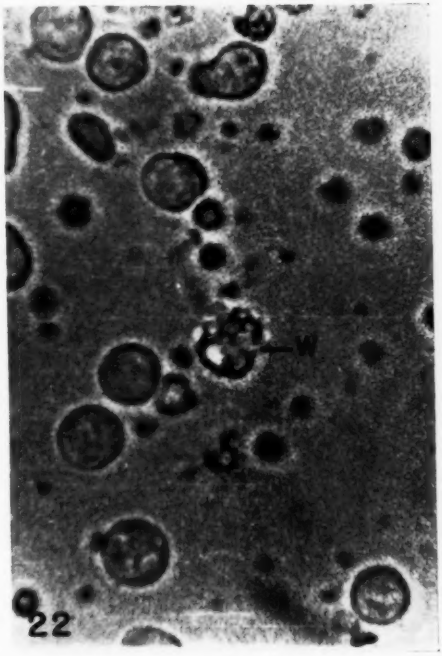
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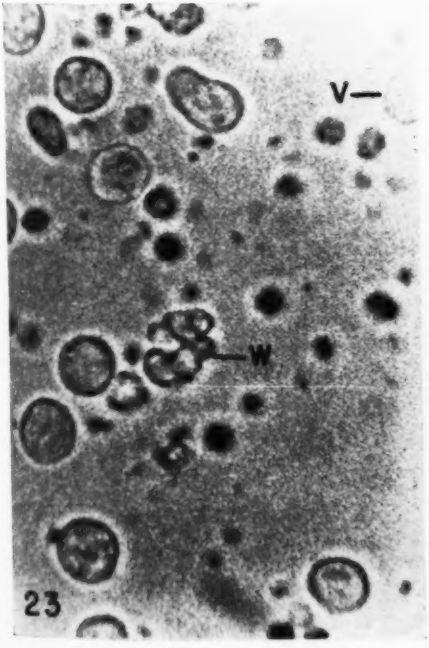

V
W
23

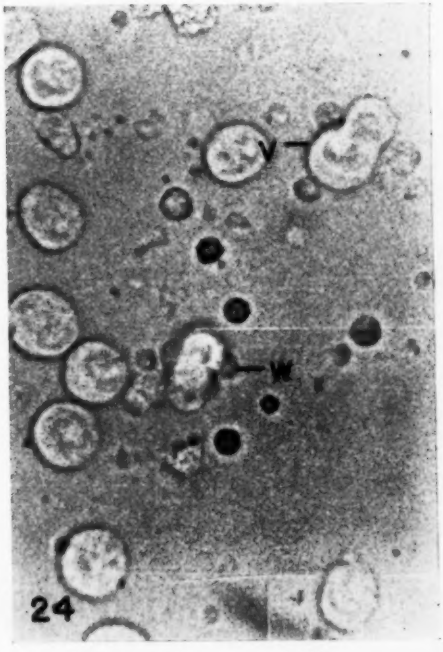

V
W
24

PLATE 95

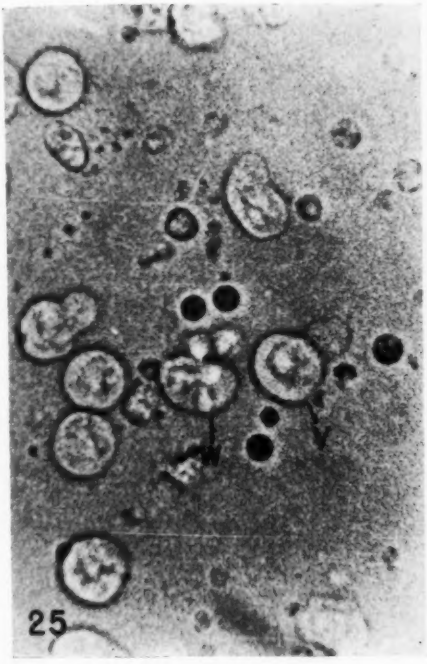
The cells shown are the same as in plate 94.

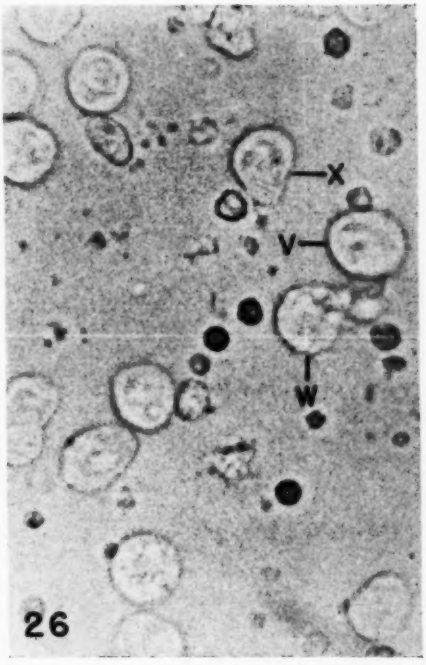
FIGURE 25.—Cell W has rounded up except for 2 small, persisting lobules. The cell is presumably dead although no definite morphologic changes can be seen with bright-field illumination. Cell V has swerved from its original path due to obstructions. Further movement in the new direction caused cell V to sideswipe cell W and the two cells became adherent to each other. Time: 12 hours, 48 minutes.

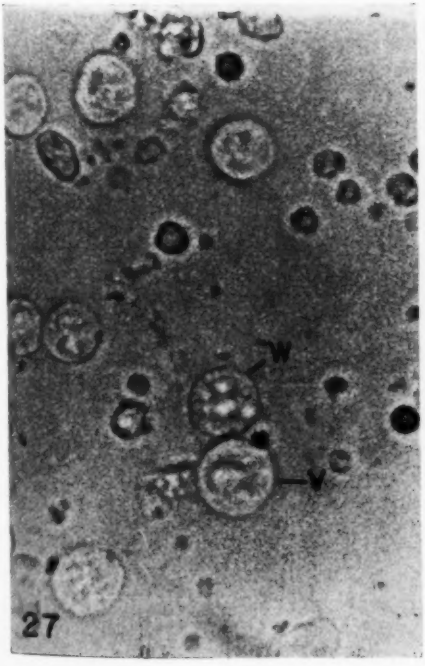
FIGURE 26.—Cell V has dragged the dead cell W from its original position. Cell V continues to be motile and is moving upward (as seen in the print) and hits first an erythrocyte and then tumor cell X. As a result of these obstructions cell V rotates 180° and starts moving downward. Time: 13 hours, 12 minutes.

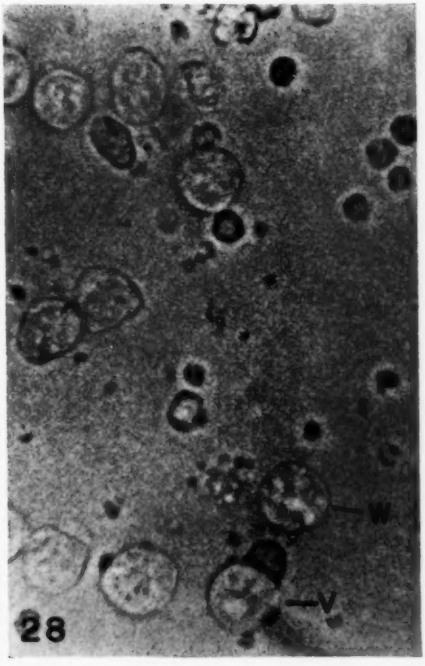
FIGURE 27.—Cell V is now moving downward and continues to pull the dead cell W behind it. A short time before this scene, cell V was caught between a red blood cell and cellular debris. The cell has just succeeded in squeezing between the two obstacles. Time: 14 hours, 26 minutes.

FIGURE 28.—Cell V is now moving diagonally out of the field. It has been moving almost continuously and has been dragging the dead cell W for about 1½ hours. The posterior tail of cell V is conspicuous. Time: 14 hours, 35 minutes.









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CONTENTS

JOURNAL OF THE NATIONAL CANCER INSTITUTE • VOL. 16 · NO. 4 · FEBRUARY 1956

Page

The chromosomes and transplantability of tumors. II. Chromosome duplication and the loss of strain specificity in solid tumors. *Leo Sachs* and *Ruth Gallily* 803

Biological studies on the Rous sarcoma virus. IV. Interpretation of tumor-response data involving one inoculation site per chicken. *W. Ray Bryan* 843

The brain of newly hatched chicks as a host-virus system for biological studies on the Rous sarcoma virus (RSV). *Vincent Groupé, Frank J. Rauscher, Alvin S. Levine,* and *W. Ray Bryan* 865

Biological studies on the Rous sarcoma virus. V. Preparation of improved standard lots of the virus for use in quantitative investigations. *John B. Moloney* 877

Chronic gastritis of the glandular stomach, adenomatous polyps of the duodenum, and calcareous pericarditis in strain DBA mice. *William V. Hare* and *Harold L. Stewart* 889

Unusual hydrolipotropic shifts in the spleen of albino rats bearing Walker carcinosarcoma 256. *Eldon M. Boyd, H. Douglas McEwen,* and *Monica E. Murdoch* 913

Influence of sex and sex hormones on development of hepatomas and other hepatic lesions in strain A × C rats ingesting 2-diacetylaminofluorene. *Harold P. Morris* and *Harlan I. Firminger* 927

Morphologic variations in an osteogenic sarcoma of the mouse when transplanted to the kidney. *Albert W. Hilberg* 951

The effects of low concentrations of carcinogen in epidermal carcinogenesis. A comparison with promoting agents. *Umberto Saffiotti* and *Philippe Shubik* 961

On the so-called "thiamin-destructive influence" by the saliva of patients with cancer or other disease. *Tatsuhei Kondo, Hideo Sakakibara,* and *Tadao Iida* 971

The effect of bacitracin on sunflower tissue cultures contaminated with bacteria. *John P. Rier, Jr.,* and *James H. M. Henderson* 981

A new, inexpensive circling device for marking specific areas on tissue smears or sections. *Pasco R. Del Vecchio, Edward E. Siegler,* and *Albert W. Hilberg* .. 985

Preliminary studies in a quantitative approach to skin carcinogenesis. *William E. Poel* and *Adolph C. Kammer* 989

The metabolism of animal tissues cultivated *in vitro*. I. Amino acid metabolism of chick embryonic-heart fibroblasts cultivated in synthetic medium M 150. *Arthur E. Pasieka, Helen J. Morton,* and *Joseph F. Morgan* 995

Effects of some metabolic inhibitors on the growth and lipide composition of Earle's strain L cells. *Relda Cailleau, Shirley Moss,* and *Benjamin V. Siegel* 1011

Toxicity of homologous immune serum to a transplantable tumor: studies using phase microscopy and cinemicrography. *Robert Schrek* and *Frederick W. Preston* 1021